Application of Stable Isotope Labeled Diphenhydramine to Study the Pharmacokinetics and Metabolism of Diphenhydramine in Pregnant, Non-Pregnant, and Fetal Sheep.

By

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II. Abstract

Diphenhydramine (DPHM), an antihistamine, has been used in pregnant women; however, information regarding its disposition in human pregnancy is lacking. Recently, detailed pharmacokinetic studies in pregnant sheep have demonstrated that DPHM readily crosses the ovine placenta, and is eliminated from the fetus by placental and non-placental pathways. The purpose of this study is to investigate the components of the fetal non-placental elimination (i.e., fetal renal and hepatic), and to compare these to the estimates obtained from adult sheep. Since stable isotope techniques were to be employed, synthesis of stable isotope labeled DPHM (i.e., \[^{2}H_{10}\]DPHM) and its major metabolite diphenylmethoxyacetic acid (i.e., \[^{2}H_{10}\]DPMA) was required. Next, gas chromatographic - mass spectrometric methods were developed to simultaneously measure either DPHM and \[^{2}H_{10}\]DPHM, or DPMA and \[^{2}H_{10}\]DPMA. The current study demonstrates that the measured fetal renal clearance of DPHM contributes only ~2% to the observed fetal non-placental clearance. Overall, the total non-placental clearance of DPHM measured by direct methods (i.e., pulmonary [Yoo, 1989] and renal) can account for ~10% of the non-placental clearance. Unlike adult sheep, where hepatic extraction of DPHM was ~93%, no significant extraction of DPHM by the fetal liver following umbilical venous administration was observed. Therefore, fetal hepatic elimination is not likely to account for the remainder of the fetal non-placental clearance. However, fetal hepatic *in vitro* metabolism of DPHM (to form N-demethyl DPHM and DPMA) suggests that the fetal liver is capable of DPHM biotransformation. Thus, the liver and possibly other organs may contribute at least a portion of the fetal non-placental clearance *via* DPHM biotransformation. It appears that only a small fraction of the fetal non-placental clearance of DPHM can be accounted for by fetal renal
and pulmonary clearances. While the low renal clearance of intact DPHM is similar both in fetus
and mother, large differences in the hepatic uptake and/or metabolism of DPHM were observed
between mother and fetus. This suggests that the pathways for the non-placental elimination of
DPHM differ in mother and fetus. Despite the advances made in this study, the components of
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Figure 50: Production of N-demethyl DPHM in fetal and maternal microsomes following a 90 minute incubation.

Figure 51: Production of DPMA in fetal and maternal microsomes following a 90 minute incubation.

Figure 52: An anatomical sketch of the fetal liver in sheep
VI List of Abbreviations

μ  Micron
α  Alpha, an exponential rate constant (apparent rate of distribution)
β  Beta, an exponential rate constant (apparent rate of elimination)
δ  Coefficient of variability estimated by maximum likelihood non-linear
curve fitting using Adapt II
γ  Coefficient of variability estimated by maximum likelihood non-linear
curve fitting using Adapt II
°C  Degree Celsius
μg  Microgram
μL  Microliter
$[^2\text{H}_{10}]\text{DPHM}$  Stable isotope (deuterium) labeled diphenhydramine
$[^2\text{H}_{10}]\text{DPMA}$  Stable isotope (deuterium) labeled diphenylmethoxyacetic acid
$^2\text{H}$  Deuterium
$^2\text{H}_2\text{O}$  Deuterium oxide
ACS  American Chemical Society
ad. lib.  ad libitum - at will
AMN  Amniotic
ANOVA  Analysis of variance
AUC  Area under the plasma concentration vs. time curve
AUMC  Area under the first moment curve
ca  Approximate
CA  Fetal carotid artery
C_{ps}  Steady-state plasma concentration
CL_{ff}  Total drug clearance from the fetal compartment
CL_{fm}  Trans-placental clearance of drug from the fetal to maternal
  compartment
CL_{fo}  Non-placental clearance of drug from the fetal compartment
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>CLmf</td>
<td>Trans-placental clearance of drug from the maternal to fetal compartment</td>
</tr>
<tr>
<td>CLmm</td>
<td>Total drug clearance from the maternal compartment</td>
</tr>
<tr>
<td>CLmo</td>
<td>Non-placental clearance of drug from the maternal compartment</td>
</tr>
<tr>
<td>cm</td>
<td>Centimeter</td>
</tr>
<tr>
<td>Cp</td>
<td>Plasma concentration</td>
</tr>
<tr>
<td>CL_T</td>
<td>Total body clearance</td>
</tr>
<tr>
<td>DOS</td>
<td>Disc Operating System</td>
</tr>
<tr>
<td>DPAA</td>
<td>Diphenylacetic acid</td>
</tr>
<tr>
<td>DPHM</td>
<td>Diphenhydramine</td>
</tr>
<tr>
<td>DPMA</td>
<td>Diphenylmethoxyacetic acid</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential scanning calorimetry</td>
</tr>
<tr>
<td>E</td>
<td>Extraction ratio</td>
</tr>
<tr>
<td>E#</td>
<td>Ewe number</td>
</tr>
<tr>
<td>ECoG</td>
<td>Electro cortical activity</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EI</td>
<td>Electronic impact ionization mode</td>
</tr>
<tr>
<td>EoG</td>
<td>Electro ocular activity</td>
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<tr>
<td>eV</td>
<td>Electron volts</td>
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<tr>
<td>F</td>
<td>Bioavailability</td>
</tr>
<tr>
<td>FA</td>
<td>Fetal femoral artery</td>
</tr>
<tr>
<td>Fm</td>
<td>Fraction of drug converted to metabolite divided by the apparent volume of distribution of the metabolite (DPMA)</td>
</tr>
<tr>
<td>fm</td>
<td>Fraction of drug converted to metabolite (DPMA)</td>
</tr>
<tr>
<td>fr.</td>
<td>French (designation of catheter sizes)</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>H¹-NMR</td>
<td>Proton nuclear magnetic resonance</td>
</tr>
<tr>
<td>H₂</td>
<td>Hydrogen gas</td>
</tr>
</tbody>
</table>
HCl  Hydrochloric acid
HP   Hewlett Packard
HPLC High performance liquid chromatograph
i.d. Internal diameter
i.e., id est; that is
IV   Intra Venous
K_{10} Apparent first-order rate constant describing the elimination of drug from
the central compartment
K_{12} Apparent first-order rate constant describing the transfer of drug from the
central compartment to the peripheral compartment in a two
compartment model
K_{21} Apparent first-order rate constant describing the transfer of drug from the
peripheral compartment to the central compartment in a two
compartment model
KCl  Potassium chloride
Kf   Apparent first-order rate constant describing the formation of metabolite
from parent drug
Kg   Kilogram
Km   apparent first-order rate constant describing the elimination of metabolite
Ko   Drug infusion rate to the mother
Ko'  Drug infusion rate to the fetus
kPa  Kilopascals
LC   Liquid chromatograph
LD_{50} Median lethal dose
LOQ  Limit of quantitation
M    Molar (moles/litre)
m    Meter
m/z  Mass to charge ratio
MA   Maternal arterial
mg   Milligram
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>MHz</td>
<td>Megahertz</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mm</td>
<td>Millimeter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MDRT</td>
<td>Mean dispositional residence time</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>MSD</td>
<td>Mass selective detector</td>
</tr>
<tr>
<td>msec</td>
<td>Millisecond</td>
</tr>
<tr>
<td>MTBSTFA</td>
<td>N-methyl-N-(tert-butyldimethylsilyl)trifluoroacetamide</td>
</tr>
<tr>
<td>MTRT</td>
<td>Mean total residence time</td>
</tr>
<tr>
<td>MV</td>
<td>Maternal femoral vein</td>
</tr>
<tr>
<td>NADH</td>
<td>β-nicotinamide-adenine dinucleotide disodium salt</td>
</tr>
<tr>
<td>NADPH</td>
<td>β-nicotinamide-adenine dinucleotide tetrasodium salt</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>NCI</td>
<td>Negative chemical ionization</td>
</tr>
<tr>
<td>ng</td>
<td>Nanogram</td>
</tr>
<tr>
<td>NPD</td>
<td>Nitrogen-phosphorous detector</td>
</tr>
<tr>
<td>o.d.</td>
<td>Outer diameter</td>
</tr>
<tr>
<td>pcg</td>
<td>Picogram</td>
</tr>
<tr>
<td>Po2</td>
<td>Partial pressure of oxygen in the blood</td>
</tr>
<tr>
<td>PCI</td>
<td>Positive chemical ionization</td>
</tr>
<tr>
<td>Pco2</td>
<td>Partial pressure of carbon dioxide in blood</td>
</tr>
<tr>
<td>PFTBA</td>
<td>Perfluorotributylamine</td>
</tr>
<tr>
<td>pH</td>
<td>Negative logarithm of hydrogen ion concentration</td>
</tr>
<tr>
<td>PFBBBr.</td>
<td>Pentafluorobenzyl bromide</td>
</tr>
<tr>
<td>P.S.I.</td>
<td>Pounds per square inch</td>
</tr>
<tr>
<td>PTFE</td>
<td>Polytetrafluoroethylene</td>
</tr>
<tr>
<td>p-TSA</td>
<td>para-toluene sulfonic acid</td>
</tr>
<tr>
<td>Qh</td>
<td>Liver blood flow</td>
</tr>
<tr>
<td>Qum</td>
<td>Umbilical blood flow</td>
</tr>
</tbody>
</table>
RBC  Red blood cells
SD   Standard deviation
SEM  Standard error of mean
SIM  Selected ion monitoring
T    Time (duration of infusion)
t    Time
T_{1/2}  Half-life
TEA  Triethylamine
TLC  Thin layer chromatography
TR   Tracheal fluid
TRIS Tris(hydroxymethyl) aminomethane
U    International units (insulin dosages)
USP  United States Pharmacopeia
UV   Umbilical Vein
V    Volts
V_c  Apparent volume of distribution of central compartment
V_{d\beta} Apparent volume of distribution
V_{d_{ss}} Apparent steady-state volume of distribution
V_m  Apparent volume of distribution of metabolite
X_g  Times gravity (centrifugal force)
\Sigma X_u  Cumulative amount of drug in urine
\Sigma M_u  Cumulative amount of metabolite in urine
VII Acknowledgments

There are a number of people without whom this project would not have been possible, and I would like to take this opportunity to thank them for their contributions to the work conducted in this thesis, and their emotional support throughout these years.

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This thesis is dedicated to the memory of my loving father Edmund Tonn. Dad, I wish that you could see me now; I hope that you are proud.
1. Introduction

1.1. General Introduction

Diphenhydramine [2-(diphenylmethoxy)-N,N-dimethylethylamine, DPHM (Molecular weight 255)] is a potent reversible H₁ receptor antagonist of the ethanolamine class (Douglas, 1985). It is a weak base with a pKa of 9.0, thus, at a physiological pH (i.e., pH 7.4) the drug is almost completely ionized. DPHM is also highly lipophilic with an octanol/water partition coefficient of 1862. DPHM (Figure 1) was one of the first H₁ receptor antagonists to be marketed in the late 1940s, and is still marketed today in Canada as either a single entity product (Benadryl® or Nytol®) or in combination with other drugs in cough and cold preparations.

![Chemical structure of diphenhydramine](image)

Figure 1: Chemical structure of diphenhydramine
1.2. Pharmacology of DPHM

DPHM binds reversibly to both peripheral and central H₁ receptors with little or no binding to H₂ receptors (Douglas, 1985). As a result, it antagonizes many of the pharmacological actions of histamine at these receptors. This includes histamine-stimulated smooth muscle contraction (e.g., gastrointestinal and respiratory smooth muscle), vasoconstriction, and, more importantly, vasodilation (Douglas, 1985). The drug also reduces vascular permeability, and thus limits edema, wheal, and flare formation following localized exposure of the dermis to histamine (Simons et al., 1990, Douglas 1985, and Bilzer et al., 1974). As a result of binding to H₁ receptors in the central nervous system (CNS), DPHM can elicit CNS depression (somnolence, slowed reaction times, and diminished alertness) at normal therapeutic doses (Douglas, 1985, Gengo et al., 1989, and Gengo, et al., 1990). In fact, at therapeutic doses somnolence has been reported in over half of the human subjects studied (Preston et al., 1992, Roehrs et al., 1993). However, at high or toxic doses, DPHM can provoke CNS stimulation (Douglas, 1985). In addition to the H₁ receptor antagonist activities, DPHM also possesses atropine-like anticholinergic, local anesthetic, antitussive, and antinausea activities (Douglas, 1985, Packman et al., 1991). Moreover, it binds to monoamine oxidases in rat and mouse brain and lung. This binding has been shown to alter the normal concentrations of various biogenic amines in these tissues (Chiavegatto and Bernardi, 1991, Shishido et al., 1991, Yoshida et al., 1989, Yoshida et al., 1990).
1.3. Toxicology of DPHM

The IV median lethal dose (LD$_{50}$) of DPHM is 42-46 mg/Kg in rats, 98 mg/Kg in mice, 10-11 mg/Kg in rabbits, and 42-46 mg/Kg in dogs, while the oral LD$_{50}$ is 500-545 mg/Kg in rats, and 164-167 mg/Kg in mice (Rieveschl and Gruhzit, 1945, and Gruhzit and Fisken, 1947). Following subcutaneous administration, the LD$_{50}$ in rats ranges from 3.8 mg/cm$^2$ in 4 day old rats to 20.7 mg/cm$^2$ in 40 day old rats, demonstrating that the LD$_{50}$ of DPHM increases with age in rats (Lee, 1966). The reason for this may be that more drug is absorbed following subcutaneous administration in younger compared to older rats (Lee, 1966). The general toxic symptoms include excitement, tremors, convulsions, and respiratory and cardiac failure (Rieveschl and Gruhzit, 1945, and Gruhzit and Fisken, 1947). In humans, DPHM has a substantial margin of safety (i.e., toxicity has been reported to occur at 6-40 times the normal therapeutic dose); however, toxicities in both adults and children are common (Douglas, 1985). Toxicity has been reported in children with chicken pox following topical DPHM administration (Chan and Wallander, 1991, Huston et al., 1990, and Bernhard and Madison, 1991). According to these reports, children were agitated, confused, and hallucinating. In more severe poisoning in children, the previously noted symptoms are often followed by convulsions, cardiovascular and pulmonary collapse, and death (Douglas, 1985). In adults, DPHM poisoning is relatively common and symptoms include sedative and anticholinergic effects, and coma. In more severe cases of poisoning, cardiovascular and pulmonary collapse and death have been reported (Clark and Vance, 1992).

DPHM, like chlorpheniramine and mepyramine, is embryo toxic, but it does not appear to be teratogenic in rats (Naranjo and de Naranjo, 1968, and Schardein et al., 1971). These data are
supported by data from retrospective human studies (Saxen, 1974). However, in combination with morphine, DPHM appears to potentiate fetal malformations (Iulliucci and Gautieri, 1971).

1.4. Clinical Applications of DPHM

DPHM is used in the treatment of various allergy-mediated diseases. It is effective in the symptomatic treatment of urticaria, seasonal rhinitis and conjunctivitis, and allergic dermatoses (Moscati and Moore, 1990, and Douglas, 1985). The drug is also marketed as an “over the counter” hypnotic. However, by virtue of DPHM’s hypnotic effects, its use for the treatment of allergy-mediated conditions is decreasing with the advent of newer non-sedating antihistamines. DPHM has also been used in various cough and cold preparations either as a single entity product or in combination with other pharmacological agents. In addition, it has been used to treat nausea and vomiting of various etiologies, including motion sickness and cancer chemotherapy (Grunberg et al., 1988, and Roila et al., 1989). Recently, DPHM has also been used as a local anesthetic agent for the repair of minor lacerations and wounds (Ernst et al., 1993).

1.5. Therapeutic Applications of Antihistamines In Pregnancy

Although the use of drugs during pregnancy is generally discouraged, certain circumstances arise where drug treatment becomes necessary. Antihistamines have been used during pregnancy for the treatment of specific pregnancy related conditions, such as pregnancy related urticaria and severe nausea and vomiting (Forfar and Nelson, 1973). However, some of
the uses of antihistamines during pregnancy are essentially extensions of their therapeutic applications in the non-pregnant population, such as the treatment of insomnia, symptomatic treatment of cough and colds, and alleviation of the symptoms of allergic rhinitis (Piper et al., 1987). A recent survey demonstrated that drugs are used in approximately 15% of pregnant women for the treatment of cold and flu, 7% for nausea and vomiting, 2% for insomnia, and 6% for skin related conditions (de Jong-van den Berg et al., 1993). Moreover, according to drug use surveys between 1963 and 1987, antihistamines were used by ~20% of pregnant women (Peckham and King, 1963, Forfar and Nelson, 1973, Hill, 1973, Doering et al., 1978, Brockelbank et al., 1978, Rayburn et al., 1982, and Piper et al., 1987), and among antihistaminic agents, DPHM was used in ~23% of the documented cases (Piper et al., 1987). These findings suggest that a significant number of human fetuses may be exposed to this drug at some time during their gestation.

1.6. Analysis Methods for DPHM

Many methods have been reported for the analysis of DPHM in biological fluids. These include UV (Wallace et al., 1966) and fluorescence (Glazko, 1974) spectrophotometry, high performance liquid chromatography (HPLC) with either UV detection (Selinger et al., 1990) or fluorescence detection (Webb and Eldon, 1991), gas chromatography (GC) analysis using flame ionization detection (Albert et al., 1974, Barni Comparini et al., 1983, and Chiarotti et al., 1983), nitrogen phosphorus specific detection (NPD) (Bilzer and Gundert-Remy, 1973, Baugh and Calvert, 1976, Abernethy and Greenblatt, 1983, Lutz et al., 1983, Meatherall and Guay, 1984, and Yoo et al., 1986), and mass spectrometry (MS) (Chang et al., 1974, Carruthers et al., 1978,
Rohdewald and Milsmann, 1986, Maurer and Pfleger, 1988, and Walters-Thompson and Manson, 1992). Although some of these methods possess the required sensitivity for the analysis of DPHM in biological fluids obtained from pregnant sheep, only methods employing MS could provide the necessary differentiation between DPHM and stable isotope labeled (SIL) DPHM. Since stable isotope techniques were to be applied, this differentiation between SIL DPHM and unlabeled DPHM was necessary. However, the GC-MS methods published to date focus on a relatively non-specific small mass fragment ion (i.e., m/z 58) (Chang et al., 1974, Carruthers et al., 1978, Rohdewald and Milsmann, 1986, Maurer and Pfleger, 1988, and Walters-Thompson and Manson, 1992). This fragment would not be capable of differentiation between DPHM and SIL DPHM, particularly if the labels were on the aromatic rings of the molecule. Therefore, development of a method capable of simultaneously measuring DPHM and SIL DPHM was required.

1.7. Pharmacokinetics of DPHM

1.7.1. Absorption of DPHM

DPHM is rapidly absorbed in humans following oral administration, with maximal plasma concentrations attained between 1-4 hours following administration (Carruthers et al., 1978, Gielsdorf et al., 1986, Blyden et al., 1986, Luna et al., 1986, and Simons et al., 1990). Peak plasma concentrations following a 50 mg oral dose were between 40 and 80 ng/mL (Carruthers et al., 1978, Gielsdorf et al., 1986, Luna et al., 1986). The drug undergoes a substantial first-pass effect following oral administration in humans, with bioavailability between
0.43 to 0.78 (Albert et al., 1975, Carruthers et al., 1978, Spector et al., 1980, and Blyden et al., 1986).

1.7.2. Distribution of DPHM

The plasma protein binding of DPHM has been reported to be as high as 98% in humans (Albert et al., 1975). However, more recent studies have suggested that the plasma protein binding of the drug is somewhat less \( (i.e., \sim 78-85\%) \) (Spector et al., 1980, Meredith et al., 1984). In addition, Spector et al. (1980) demonstrated that there is a difference in the plasma protein binding between Orientals and Caucasians \( (i.e., \text{binding was } 76\% \text{ in Caucasians and } 85\% \text{ in Orientals}) \). It has also been demonstrated that the binding of DPHM decreases from 78% to 67% in chronic liver disease (Meredith et al., 1984). Since binding of DPHM to human serum albumin is only \( \sim 30\% \) (Drach et al., 1970), it is likely that other plasma proteins, such as \( \alpha\)-1-acid glycoprotein (known to bind basic drugs) may also play a role in the binding of this agent (Kremer et al., 1988). However, the binding of DPHM has not been extensively studied in either humans or animals. The tissue distribution pattern following either oral, intraperitoneal, and intravenous administration to either rats or guinea pigs showed that the highest tissue drug concentrations were in the lung, followed by the spleen, brain, liver, muscle, and heart (Glazko and Dill, 1949a). The high tissue distribution of DPHM in the lung may be due to the high binding of this drug to monoamine oxidases, as demonstrated in perfused rat lung and in isolated rat lung mitochondria (Yoshida et al., 1989 and 1990).
1.7.3. Metabolism of DPHM

In rat, guinea pig, and rabbit, DPHM is extensively degraded in liver homogenates, and to a lesser extent in lung and kidney homogenates (Glazko and Dill, 1949b). The degradation in rat liver appears to be rapid, with approximately 75% of the added amount of DPHM degraded within 120 minutes. Similar findings were reported by Lee (1966), who demonstrated that DPHM degradation occurs rapidly, and that the rates did not differ between hepatic homogenates prepared from older (40 days) and younger (15 days) rats. The large first-pass effect following oral administration and the reduced clearance of DPHM in patients with chronic liver disease suggests that hepatic elimination of DPHM may also be an important route of elimination in humans (Meredith et al., 1984, Albert et al., 1975, Carruthers et al., 1978, and Blyden et al., 1986). The hepatic elimination of this drug may partially occur via N-demethylation, since DPHM was found to be rapidly N-demethylated in rat liver microsomes (Roozemond et al., 1965). DPHM and some closely related analogs (i.e., orphenadrine) form metabolic-intermediate complexes with the rat liver cytochrome P450 2B1 and 2C6 isoforms (Bast et al., 1990, Rekka et al., 1989, and Reidy et al., 1989). The formation of these complexes may inhibit the metabolism, and thus, the elimination of other drugs. Recently Hussain et al. (1994) demonstrated that the co-infusion of DPHM with diltiazem in perfused rat livers resulted in an initial sharp increase in the diltiazem perfusate concentration, followed by 45% higher steady-state concentrations of diltiazem compared to control values. This was thought to be due to both displacement of diltiazem from hepatic tissue binding sites and the inhibition of diltiazem metabolism by DPHM (Hussain et al., 1994). DPHM has also been shown to undergo N-deamination in hepatic microsomes prepared from rats, guinea pigs, and rabbits (Yamada et al.,
These studies demonstrated that DPHM was deaminated to form methylamine, suggesting that the drug must be demethylated prior to being deaminated (Yamada et al., 1993).

In humans, rhesus monkeys, and dogs, the metabolites identified \textit{in vivo} suggest that DPHM undergoes successive N-demethylation to give N-demethyl DPHM and N,N-didemethyl DPHM, followed by deamination to yield diphenylmethoxyacetic acid (Drach and Howell, 1968, Chang et al., 1974, Glazko et al., 1974, and Drach et al., 1970). Diphenylmethoxyacetic acid (DPMA, Molecular weight 242) has been demonstrated to form glycine conjugates in dogs and glutamate conjugates in rhesus monkeys (Drach and Howell, 1968, Drach et al., 1970). DPMA and its conjugates are the most prominent urinary metabolites identified in dogs (~42%), and rhesus monkeys (~60%) (Drach et al., 1970). In addition, significant quantities (\textit{i.e.}, 10-20%) of the N-oxide metabolite of DPHM were found in the urine in all species examined to date (Drach and Howell, 1968, Drach et al., 1970, Chang et al., 1974). The \textit{in vivo} metabolism of DPHM in the rat has not yet been completely established, and many of the metabolites in this species remain unidentified (Drach and Howell, 1968). Recently, DPHM was shown to form a quaternary ammonium glucuronide conjugate in humans (Luo et al., 1991 and 1992). In humans, Glazko et al. (1974) and Blyden et al. (1986) demonstrated that plasma concentrations of the N-demethylated metabolites declined in a fashion similar to intact DPHM. However, DPMA was found to accumulate in plasma for up to 24 hours following the oral administration of DPHM, and exceed plasma concentrations of DPHM and N-demethyl DPHM by \textasciitilde 10 fold (Glazko et al., 1974).
1.7.4. **Excretion of DPHM and its Metabolites**

Only a small fraction of the DPHM dose administered is excreted as intact drug in the urine of rats (≈ 4-6%), rabbits (<3%), dogs (≈4%), and monkeys (≈3%) (Glazko et al., 1949, Drach et al., 1970, and Parry and Calvet, 1982). Similarly, only a small portion (2-4%) of intact DPHM is excreted in humans (Albert et al., 1975, Meredith et al., 1983). However, a large portion of DPHM’s metabolites are excreted in urine. The percentage of urinary metabolites excreted is ≈35% of the dose in rats, and ≈49% in humans (Glazko et al., 1949, and Drach et al., 1970). The role of biliary excretion of DPHM and/or its metabolites has not yet been reported in either laboratory animals or humans.

1.8. **DPHM Disposition in Pregnancy**

With the exception of one case, which documented neonatal DPHM withdrawal symptoms, information regarding DPHM effects and disposition in human pregnancy is absent (Parkin, 1974). Due to ethical and technical constraints, detailed studies of DPHM pharmacokinetics and pharmacodynamics in pregnancy cannot be conducted in humans. Therefore, several approaches have been developed to assess the extent of fetal exposure and placental transfer of drugs. Single point estimations of drug concentrations in cord blood from humans following birth provide clinically relevant information; however, these estimates are highly dependent on the time of sampling relative to that of drug administration (Anderson et al., 1980, and Levy and Hayton, 1973). The perfused human placenta has also been used to detail the placental transfer of drugs, however, the development of leaks in the placenta during perfusion can provide misleading findings (Faber and Thornburg, 1983). Several small animal
species, such as rats, guinea pigs, and rabbits have been utilized to examine drug disposition in the fetal/maternal unit. However, a limitation of this approach is that serial blood samples cannot be obtained due to the small fetal blood volume in these species (Rurak et al., 1991). Chronic catheterized preparations, either sheep or primates, have been utilized for detailed maternal and fetal pharmacokinetic and pharmacodynamic studies (Rurak et al., 1991). Sheep are the most commonly employed species; however, they possess an epithelialchorial placenta which is less permeable to hydrophilic endogenous compounds and drugs, unlike the hemochorial placenta found in humans, primates, and several small animal models (Rurak et al., 1991, and Faber and Thornburg, 1983). Therefore, fetal/maternal pharmacokinetic experiments conducted in sheep with polar hydrophilic compounds may not provide quantitative data that is relevant to the situation in humans (Rurak et al., 1991). However, the placental transfer of hydrophobic compounds, such as DPHM, does not appear to be limited in the sheep placenta (Yoo et al., 1986, Rurak et al., 1991).

1.8.1. Disposition and Fetal Effects of DPHM in Pregnant Sheep

Yoo et al. (1986) demonstrated that DPHM undergoes rapid placental transfer to the fetal lamb, with maximum fetal levels occurring within 5 minutes following maternal bolus administration. There was also extensive fetal exposure to the drug following maternal bolus administration (i.e., AUC fetal/AUC maternal = 0.85). The lipophilic nature of the drug and the rapid and extensive exposure of the fetus following maternal administration suggests that transplacental transfer of DPHM occurs by simple diffusion. Further, it was shown that DPHM does not persist in the fetal circulation, since the apparent terminal elimination half-life was similar in
both mother and fetus (i.e., 52 vs. 46 minutes, respectively). Similar to other amine drugs, DPHM was found to accumulate in fetal tracheal and amniotic fluids, with levels in the tracheal fluid four fold greater than fetal plasma concentrations (Yoo, 1989, Riggs, et al., 1987, and Rurak et al., 1991). The administration of DPHM via the amniotic cavity resulted in preferential fetal uptake (Rurak et al., 1994). The routes of the fetal drug uptake identified are fetal swallowing, and uptake via the fetal membranes. These data suggest that DPHM present in the amniotic fluid could be recirculated in the fetal lamb via these mechanisms (Yoo et al., 1989).

Time-separated fetal and maternal infusions to steady-state, utilizing a two compartment-open model (Szeto et al., 1982), demonstrated that both mother and fetus can eliminate DPHM via placental and non-placental pathways (Yoo et al., 1993). In the fetal lamb, the rate of non-placental clearance was approximately three fold greater than that observed in the mother on a weight corrected basis (Yoo et al., 1993). This suggests that the fetal lamb, per Kg, is more efficient in eliminating the drug than the mother. The elimination of DPHM via the fetal lung, although resulting in high tracheal fluid levels, accounts for only a small portion (~8%) of the fetal elimination of the drug (Rurak et al., 1991). To date, this is the only specific route of fetal non-placental elimination that has been explained for DPHM. Thus, for this drug, the bulk of the non-placental clearance remains to be elucidated. DPHM is not unique in this regard. Other drugs, including ritodrine, labetalol, acetaminophen, metoclopramide, meperidine, and morphine all undergo substantial fetal non-placental clearance (Wright et al., 1991, Yeleswaram et al., 1993, Wang et al., 1986, Riggs et al., 1990 and Szeto et al., 1982), yet the components of their non-placental clearance remain largely unknown. Wang et al. (1986) demonstrated that ~97% of the maternal non-placental clearance could be accounted for by metabolic and renal pathways; however, in the fetal lamb only ~33% of the non-placental clearance of acetaminophen could be
accounted for by these pathways. Olsen et al. (1988) also demonstrated that only ~63% of
morphine infused to the fetus resulted in the formation of morphine-3-glucuronide. Similar
results were also noted for ritodrine and labetalol (Wright et al., 1991, and Yeleswaram et al.,
1993). Thus, an understanding of the fetal non-placental components responsible for DPHM
elimination may also provide some insight into the non-placental clearance of other drugs.

Yoo et al. (1993) also demonstrated that a difference exists for DPHM trans-placental
clearances from fetus to mother (CLfm) and from mother to fetus (CLmf). The CLfm was 2-3
fold greater than the CLmf. Despite correcting these clearance estimates (i.e., CLmf and CLfm)
for differences between the fetal and maternal plasma protein binding (i.e., ~86% is bound in the
ewe and only 72% in the fetal lamb), the differences, although somewhat lower, still remained.
The reason for this phenomenon is not currently known, however, the magnitude of the greater
difference in CLfm compared to CLmf correlates with greater drug lipophilicity, and thus,
placental permeability (CLfm) (Yoo et al, 1993).

Ideally, with the 2-compartment-open model employed by Yoo et al. (1993), the fetal and
maternal infusions should be conducted simultaneously; however, without labeled drug, time-
separated infusions are required (Szeto, 1982, Szeto et al., 1982, Yoo et al., 1993). Due to the
rapid elimination of DPHM from both mother and fetus, only a 48 hour washout period was
required in these studies. The effects of this washout period on the disposition of DPHM in the
dynamic fetal/maternal unit are not clear; however, the short washout period would have likely
minimized these effects.

During both fetal and maternal infusions, DPHM elicits substantial fetal behavioral
effects in sheep. These fetal effects appeared to vary in relation to the fetal plasma
concentrations achieved. Rurak et al. (1988) demonstrated that at lower fetal drug
concentrations (~36 ng/mL) achieved with maternal drug administration, the fetal effects were consistent with CNS depression (i.e., decreases in low voltage ECoG pattern, low voltage ECoG patterns associated with rapid eye movements, and the overall incidence of fetal breathing movements). At higher plasma concentrations achieved with fetal drug infusion (~448 ng/mL), transient declines in Po2 and pH, associated with tachycardia and vigorous fetal breathing movements were observed during the initial portion of the infusion. In addition, there was a fall in low voltage ECoG activity and a marked increase in intermediate voltage ECoG pattern (Rurak et al., 1988).

1.9. Stable Isotopes

The past two decades have seen a steady rise in the use of stable isotope labeled compounds to investigate drug pharmacokinetics and metabolism in both laboratory animals and man (Browne, 1990). Stable isotopes are, as the name implies, stable forms (non-radioactive) of an atom which differ only in atomic mass due to differing numbers of neutrons in the nucleus. Stable isotope labeling refers to the substitution of an atom in a molecule of interest with its corresponding stable isotope (e.g., $^{13}$C, $^{17}$O, $^{18}$O, $^{15}$N, and $^2$H). In most cases, the stable isotope labeled (SIL) analog of the original molecule will have identical physical and chemical properties to the unlabeled molecule, but will differ in molecular mass. The most widely used analytical methodology to differentiate between SIL molecules and their unlabeled counterparts employs mass spectrometry coupled with either GC or HPLC (Baillie, 1981). To enhance the selectivity, and thus discern between labeled and unlabeled drug, the mass spectrometer is run in the selective ion monitoring mode (SIM). This simply means that the mass spectrometer is
programmed to focus on individual fragment ions for the SIL drug and the unlabeled drug. Thus, SIM can provide both the necessary differentiation between a SIL and an unlabeled molecule (selectivity) and the required sensitivity (sub-nanogram range) for maternal and fetal pharmacokinetic studies.

The use of SIL compounds in pharmacokinetic experiments provides several advantages over traditional experimental designs (Browne, 1990, and Baillie, 1981). The simultaneous co-administration of SIL and an unlabeled counterpart in pharmacokinetic studies essentially allows two experiments to be conducted on one occasion (i.e., the control and the test). This significantly reduces the inter-day variability and the influence of time dependent changes on pharmacokinetic parameters (Baillie, 1981, Browne, 1990, and Eichelbaum, 1982). In addition, this technique can also reduce the number of exposures to the drug, samples to be analyzed, and number of experimental days (Browne, 1990). These advantages essentially translate into a reduction in the number of subjects/animals required for the equivalent degree of statistical power, and a reduction in cost and time.

The key advantage of using stable isotope techniques in the study of drug disposition in pregnancy is the elimination of possible time-dependent effects on pharmacokinetic parameters due to the dynamic nature of the fetal/maternal unit (Battaglia and Meschia, 1986). An increase in the statistical power resulting in a possible reduction in the number of study subjects is also of paramount importance since the acquisition, preparation, and maintenance of chronically instrumented pregnant sheep and other similar preparations (e.g., primates) is very costly. In addition, this approach allows for better utilization of animal resources. Since two experiments can be conducted on one occasion, more experiments can be conducted on one animal during the narrow time window available for experimentation (i.e. 7-21 days) before the ewe delivers. By
the same virtue, conducting both the control and test experiments on one occasion increases the probability of conducting successful experiments (i.e., there is less loss of experimental data due to catheter failures or fetal death occurring between test and control administrations than when using only unlabeled drug).

There are also limitations to the use of stable isotope techniques. The largest impediment is the lack of accessibility to SIL technology (i.e., instrumentation, SIL drug, and analytical methodology) (Browne, 1990). Moreover, the key assumption made following the simultaneous co-administration of the SIL and the unlabeled compound is that the SIL compound displays "equivalent" disposition characteristics to the unlabeled compound (i.e., absorption, distribution, metabolism, and excretion) (Baillie, 1981, Van Langenhove, 1986, Browne, 1990, and Chasseaud and Hawkins, 1990). If this is not the case, the resulting "isotope effect" (i.e., the difference between the labeled and the unlabeled drug) would severely limit the utility of the SIL drug for pharmacokinetic studies. Therefore, prior to conducting an experiment utilizing the simultaneous administration of SIL and unlabeled drug, the absence of an isotope effect must be verified (Wolen, 1986, Baillie, 1981, and Van Langenhove, 1986). However, where possible, it is also important to investigate the metabolic profile following administration of the SIL and unlabeled drug to ensure that the observed pharmacokinetic equivalence of the intact drug also corresponds to the metabolites generated (i.e., to rule out possible metabolic shifting) (Eichelbaum et al., 1990).

1.10. Rationale and Objectives

The investigation of DPHM disposition in pregnant sheep has demonstrated that the drug rapidly and readily crosses the ovine placenta. In addition, these studies have shown that it is
eliminated by both placental and non-placental means, and that the weight corrected fetal non-
placental clearance of DPHM exceeds that of the adult by 3 fold. The components of this large
fetal non-placental clearance of DPHM is not clear, since only ~8% of the fetal non-placental
clearance can be accounted for (fetal pulmonary uptake). This situation is not unique to DPHM;
that is, for all drugs that have been studied in sheep, the routes of fetal non-placental clearance
have not been fully elucidated. Moreover, for some compounds it is clear that the routes of
elimination in the mother cannot account fully for fetal clearance of the drug. Thus, accounting
for the remainder of the fetal non-placental clearance of DPHM may provide a more general
insight into drug disposition in the fetal lamb. In addition, the elimination of DPHM by fetal
non-placental means could involve fetal and/or placental drug metabolism, and result in the
formation of possible active and/or toxic metabolites which may distribute, accumulate, and
possibly even persist in the fetus. This further necessitates investigations into the fetal non-
placental clearance of DPHM. However, since the metabolism of DPHM has not yet been
documented in sheep, studies examining the elimination of the drug in adult sheep must be
conducted to provide comparative data. Therefore, the objectives of the current study were to
examine and contrast the fetal and maternal hepatic and renal contribution towards the
elimination of DPHM in chronically instrumented pregnant sheep. Since stable isotope
techniques have distinct advantages for the study of pharmacokinetics in the maternal/fetal unit,
they were used in the current study. However, prior to the application of stable isotope
techniques, a labeled analog of DPHM must be synthesized, and an analytical method developed
which could simultaneously measure DPHM and SIL DPHM. In addition, the absence of an
isotope effect for SIL DPHM must be verified in adult and fetal sheep. Once these tasks have
been completed, the components of the fetal and maternal non-placental clearance can be investigated.

1.11. **Hypothesis and Specific Aims**

The working hypothesis of this thesis was:

Hepatic elimination of DPHM and renal excretion of the drug and its metabolites contribute significantly to the overall non-placental clearance of DPHM in the fetal lamb.

To test this hypothesis, the specific aims of this project were to:

1. Synthesize a stable isotope analog of DPHM, namely, $[^{2}\text{H}_{10}]$DPHM, and to develop a sensitive and specific GC-MS method for the simultaneous quantitation of DPHM and $[^{2}\text{H}_{10}]$DPHM.

2. Synthesize a stable isotope analog of a prominent DPHM metabolite (*i.e.*, DPMA), namely $[^{2}\text{H}_{10}]$DPMA, and to develop a sensitive and specific GC-MS method for the simultaneous quantitation of DPMA and $[^{2}\text{H}_{10}]$DPMA.

3. Test for the presence of isotope effects in the disposition of $[^{2}\text{H}_{10}]$DPHM in maternal sheep following bolus administration, and fetal sheep following both bolus administration and fetal infusion.
4. Apply stable isotope methodology to determine the hepatic first-pass metabolism of DPHM in non-pregnant adult sheep following mesenteric (portal venous) bolus administration.

5. Apply stable isotope methodology to determine the hepatic first-pass metabolism of DPHM in fetal lambs following umbilical venous bolus administration and infusion.

6. Utilize simultaneous maternal and fetal infusions of DPHM and [\(^{2}\text{H}_{10}\)]DPHM to measure trans-placental and non-placental clearances in the ovine fetal/maternal unit, respectively.

7. Use stable isotope techniques to characterize the disposition of DPMA in both maternal and fetal sheep following simultaneous maternal/fetal infusions of DPHM and [\(^{2}\text{H}_{10}\)]DPHM, respectively.

8. Calculate the contribution of DPHM and [\(^{2}\text{H}_{10}\)]DPHM, DPMA, and [\(^{2}\text{H}_{10}\)]DPMA renal elimination towards the measured non-placental maternal and fetal clearances.

9. Assess the fetal behavioral effects following simultaneous infusions of DPHM and [\(^{2}\text{H}_{10}\)]DPHM to mother and fetus, respectively.

10. Compare the metabolism of DPHM in hepatic microsomes prepared from fetal and adult sheep.
2. Experimental

2.1. Materials

Reference standards, chemicals, reagents and other materials used during this thesis project are listed below, along with information on purity (where applicable), and the source. Unless otherwise specified, the materials were used without prior purification or modification. The materials utilized were: diphenhydramine hydrochloride [2-(diphenylmethoxy)-N,N-dimethylethylamine] (>99% purity), orphenadrine hydrochloride [N,N-dimethyl-2-[(2-methylphenyl)phenyl-ethylamine] (>99% purity), diphenylacetic acid (> 98% purity)(Sigma Chemical Co., St. Louis, MO, U.S.A.); thiopental sodium injectable 1 g/vial; sodium chloride for injection USP (Abbott Laboratories, Montreal, Que.); injectable ampicillin (250 mg/vial) (Novopharm, Toronto, Ont.); injectable gentamicin sulfate (40 mg/vial) (Schering Canada, Ltd, Pointe Claire, Que.); injectable atropine sulfate (0.6 mg/mL) (Glaxo Laboratories, Montreal, Que.); heparin 1000 units/mL (Organon Canada Ltd., West Hill, Ont.); halothane (Ayerst Laboratories, Montreal, Que.); lidocaine 2% (Astra Pharma Inc., Mississauga, Ont.). All injectable drug formulations were obtained from the Pharmacy Department, Grace Hospital, Vancouver, B.C.

Other materials used during the course of this project were: deuterated benzene ([^2]H_6 benzene, 99.5% purity) (MSD Isotopes, Montreal, Que.); anhydrous aluminum chloride, anhydrous sodium sulfate, anhydrous magnesium sulfate, bromoacetic acid, carbon tetrachloride, diethyl ether, disodium hydrogen orthophosphate (dibasic, ACS reagent grade), ethyl alcohol, hydrochloric acid, isopropyl alcohol, magnesium chloride, HPLC grade methanol, petroleum
ether, potassium dihydrogen orthophosphate (monobasic, ACS reagent grade), potassium chloride, sodium metal, sodium hydroxide pellets (ACS reagent grade), and *para*-toluene sulfonic acid (BDH, Toronto, Ont.); deuterium oxide (99.9% purity) (Aldrich Chemical Co., Milwaukee, WI, U.S.A.); triethylamine (TEA), N-methyl-N-(*tert-*butyldimethylsilyl) trifluoroacetamide (MTBSTFA) and pentafluorobenzyl bromide (PFBBBr) (sequanal grade) (Pierce Chemical Co., Rockville, IL, U.S.A.); ethyl acetate, n-hexane, methylene chloride, and toluene all distilled in glass (Caledon Labs., Georgetown, Ont.); ethylenediaminetetraacetic acid (EDTA), sucrose, tris[hydroxymethyl]aminomethane (Trizma® Base) (Sigma Chemical Co., St. Louis, MO, U.S.A.); β-nicotinamide-adenine dinucleotide (reduced) disodium salt *ca.* 98% (NADH), and β-nicotinamide-adenine dinucleotide phosphate (reduced) tetrasodium salt *ca.* 98% (NADPH) (Boehringer Mannheim Canada, Laval, Que.).

Deionized, high purity water was produced on-site by reverse osmosis and subsequent filtration using a Milli-Q® water system (Millipore, Bedford, MA, U.S.A.). This water will be referred to as distilled water in the remaining sections of this thesis.

Ultra-high purity helium, hydrogen, and zero air (Matheson Gas, Edmonton, Alta.), and nitrogen USP (Union Carbide Canada Ltd., Toronto Ont.) were utilized.

Also used were: needles and plastic disposable Luer-Lok® Syringes for drug administration and sample collection (Becton-Dickinson Canada, Mississauga, Ont.); disposable plastic pipette tips (National Scientific Supply Company, Inc., San Rafael, CA, U.S.A.); nylon syringe filters (0.22μ) (MSI, Westboro, MA, U.S.A.); borosilicate glass pasteur pipettes (Johns Scientific, Toronto, Ont.); heparinized blood gas syringes (Marquest Medical Products Inc., Englewood, CO, U.S.A.); heparinized Vacutainer® tubes (Vacutainer Systems, Rutherford, NJ, U.S.A.); 15 mL Pyrex® disposable culture tubes (Corning Glass Works, Corning, NY, U.S.A.);
polytetrafluoroethylene (PTFE) lined screw caps (Canlab, Vancouver, B.C.); Silicone rubber
tubing for catheter preparation (Dow Corning, Midland, MI, U.S.A.); PTFE-coated stainless steel
wire for electrode preparation (Cooper Corp., Chatsworth, CA, U.S.A.); cellophane dialysis
membrane “sacks” (molecular weight cutoff = 12,000 daltons) (Sigma Chemical Co., St. Louis,

2.1.1. Preparation of Stock Solutions and Buffers

Standard stock solutions of diphenhydramine (DPHM) and \([^2\text{H}_{10}]\)diphenhydramine
\(([^2\text{H}_{10}]\text{DPHM})\) were prepared with accurately weighed portions of DPHM hydrochloride (HCl)
and \([^2\text{H}_{10}]\text{DPHM HCl}\). These weighed portions were dissolved in distilled water and diluted in a
serial fashion to yield final concentrations of 200.0 ng/mL and 208.0 ng/mL (mass corrected for
the mass of the stable isotope label) of DPHM and \([^2\text{H}_{10}]\text{DPHM free base}\), respectively. The
stock solution of orphenadrine, the internal standard for the DPHM/\([^2\text{H}_{10}]\text{DPHM}\) assay, was
prepared in a similar fashion to yield a final concentration of 1.0 \(\mu\)g/mL. The aqueous standard
solutions of diphenylmethoxyacetic acid (DPMA) and \([^2\text{H}_{10}]\)diphenylmethoxyacetic acid
\(([^2\text{H}_{10}]\text{DPMA})\) were prepared with distilled deionized water to yield final concentrations of
500.0 ng/mL and 520.0 ng/mL (mass corrected for the mass of the stable isotope label),
respectively. Diphenylacetic acid (DPAA), the internal standard for the DPMA/\([^2\text{H}_{10}]\text{DPMA}\)
assay, was initially dissolved in methanol. An aliquot of the methanolic solution was diluted
with distilled deionized water to give a final concentration of 2.0 \(\mu\)g/mL. All stock solutions
were stored at 4°C, and were used for no longer than six months. There was no evidence of
degradation of these standard stock solutions during this time period. Standard solutions of all
the above analytes were also prepared in methanol for extraction recovery studies. These solutions were prepared and used on the same day to prevent evaporation of the methanol.

A solution of TEA in toluene (0.0125M) was prepared by diluting TEA with toluene distilled in glass. Four to five pellets of sodium hydroxide (NaOH) were added to the solution to ensure dryness. This solution was stored at 4°C.

A 1.0 M NaOH solution was prepared by dissolving sodium hydroxide pellets with distilled water, and hydrochloric acid (1.0 M) was prepared by diluting ACS reagent grade concentrated HCl acid with distilled water.

Isotonic phosphate buffer (0.1 M, pH 7.4) was prepared from accurately weighed portions of potassium phosphate (mono-basic), disodium phosphate, and sodium chloride. If necessary, the pH was adjusted to pH 7.4 with small aliquots of 1.0 M NaOH or HCl.

The 0.05 M Tris-HCl: 1.15% potassium chloride (KCl) [pH 7.4 at 4°C] buffer was prepared by dissolving Trizma® base and KCl with cooled distilled water (4°C). The pH was adjusted using 1.0 M HCl. The 10 mM EDTA: 1.15% KCl (pH 7.4) buffer solution was prepared by dissolving the appropriate quantities of EDTA and KCl with distilled water (4°C). The pH was adjusted with 1.0 M HCl. A 0.25 M sucrose solution was prepared by dissolving a weighed portion of sucrose with distilled water. These solutions were stored at 4 °C and used prior to 3 months in the case of the Tris-KCl and EDTA-KCl buffers. The sucrose buffer was prepared on a monthly basis.
2.2. **Equipment and Instrumentation**

2.2.1. **Gas Chromatography with Nitrogen Phosphorus Specific Detection**

A Hewlett Packard (HP) model 5890 (Series II) gas chromatograph was equipped with a split-splitless capillary inlet system, a HP Model 7673 autoinjector, a nitrogen-phosphorus specific detector, a Vectra\textsuperscript{®} 386 SX computer equipped with HP 3365 MS DOS\textsuperscript{®} workstation software (Version A.02.01), a cross-linked fused silica capillary column (25 m X 0.31 mm i.d., film thickness, 0.25\(\mu\), 5\% phenylmethylsilicone) (Ultra-2), a 4 X 78 mm borosilicate glass inlet liner (Hewlett Packard Ltd., Avondale, PA, U.S.A.); and a silicone rubber septa (Thermogreen LB-2\textsuperscript{®}, Supelco, Bellafonte, CA, U.S.A.).

2.2.2. **Gas Chromatography with Mass Spectrometry**

A HP model 5890 (Series II) gas chromatograph was equipped with a split-splitless capillary inlet system, a HP Model 7673 autoinjector, a HP Model 5971A quadrapole mass selective detector, and a Vectra 486 25T Computer equipped with MS DOS\textsuperscript{®} HP Model G1030A workstation software, a cross-linked fused silica capillary column (25 m X 0.31 mm i.d., film thickness, 0.25\(\mu\), 5\% phenylmethylsilicone; HP Ultra-2; DPMA/[\textsuperscript{2}H\textsubscript{10}]DPMA assay method, or DB-1701 30 m X 0.25 mm i.d., 0.25 \(\mu\), 5\% phenylmethylsilicone and 7\% cyanopropylsilicone; J&W Scientific, Folsom, CA, U.S.A.; DPHM/[\textsuperscript{2}H\textsubscript{10}]DPHM assay method), a 4 X 78 mm borosilicate glass inlet liner, and a Thermogreen LB-2\textsuperscript{®} silicone rubber septa.
2.2.3. **Gas Chromatography/High Performance Liquid Chromatography - Mass Spectrometry**

A HP Model 5989 MS Engine consisting of a HP 5890 Series II gas chromatograph with a HP 5989 quadrapole mass spectrometer capable of negative chemical (NCI), positive chemical (PCI) and electron impact (EI) ionization modes, and a HP 1090 microbore high performance liquid chromatograph was employed. For LC/MS, thermospray sample introduction was used. LC/MS analysis was conducted using direct flow injections via the thermospray interface. (i.e., no LC column was used). The carrier phase was composed of 50% ammonium acetate buffer 10 mM pH 7.0: 50% acetonitrile. The thermospray capillary temperature was maintained at 120 °C, and the fragmenter was turned off. The ion source was run in the positive ion scanning mode (Mass Range m/z 120 to 400).

2.2.4. **Spectrophotometer**

A HP 8452A diode array spectrophotometer equipped with a Vectra® computer interface was used for all spectrophotometric measurements.

2.2.5. **Physiological Monitoring**

A Beckman R-711 Dynograph Recorder (Beckman Instruments, Inc., Palo Alto, CA, U.S.A.) was equipped with disposable DTX transducers (Spectramed, Oxnard, CA, U.S.A.), cardiotachometers (Model 9857, Sensormedics, Anaheim, CA, U.S.A.), and transit-time blood flow transducers (Transonic Systems Inc., Ithaca, NY, U.S.A.). An Apple IIe computer and
computer data acquisition system consisting of an interactive systems analog to digital converter (Daisy Electronics, Newton Square, PA, U.S.A.), and a clock card (Mountain Software, Scott's Valley, CA, U.S.A.) were connected in series with the recorder. Blood chemistry measurements were made with an IL 1306 pH/Blood gas analyzer (Allied Instrumentation Laboratory, Milan, Italy), a Hemoximeter (Radiometer, Copenhagen, Denmark), and a 2300 STAT plus glucose/lactate analyzer (Y.S.I. Inc. Yellow Springs, OH, U.S.A.).

2.2.6. General Experimental Equipment

Other equipment utilized included: a vortex-type mixer (Vortex-Genie), and an incubation oven (Isotemp model 350) (Fisher Scientific Industries, Springfield, MA, U.S.A.); an IEC model 2K centrifuge (Damon/IEC division, Needham Hts., MA, U.S.A.); a rotating-type mixer (Labquake model 415-110, Lab Industries, Berkeley, CA, U.S.A.); an infusion pump (Harvard model 944, Harvard Apparatus, Millis, MA, U.S.A.); a DIAS Roller pump (DIAS, Ex154, DIAS Inc. Kalamazoo, MI, U.S.A.); a $^1$H-NMR (Bruker AC-200 [200MHz], U.B.C. Chemistry Department); a differential scanning calorimeter (Series 99 Thermal Analyzer, Dupont Clinical Instruments, Wilmington, DE, U.S.A.); a high speed centrifuge model J2-21, an ultra-centrifuge model L8-60M or L5-50, JA-17 fixed angle rotor, Ti 50.2 fixed angle rotor (Beckman Instruments, Inc., Palo Alto, CA, U.S.A.).
2.3. Chemical Synthesis of Standards and Metabolites of DPHM

2.3.1. Synthesis of $[^2\text{H}_{10}]$benzhydrol

Stable isotope labeled benzhydrol was synthesized in two steps (Figure 2). The initial step involved the synthesis of $[^2\text{H}_{10}]$benzophenone. This method was similar to a method for the synthesis of unlabeled benzophenone (Marvel and Sperry, 1941). Into a three neck 250 mL Pyrex® round-bottom flask equipped with a Teflon® coated magnetic stirring rod, 7.6 g anhydrous aluminum chloride and 16.7 mL of dry carbon tetrachloride were added. A thermometer, a separatory funnel, and a reflux condenser equipped with a water and HCl trap were added onto the round bottom flask. The reaction was started with the addition of 0.8 mL of $[^2\text{H}_6]$benzene. The reaction mixture was cooled using an ice bath to maintain the temperature in the reaction flask between 10-15° C. A mixture of $[^2\text{H}_6]$benzene (9.2 mL) and carbon tetrachloride (9.2 mL) was added in a drop-wise fashion to the reaction mixture from the separatory funnel (2-3 hours). Following the addition of the $[^2\text{H}_6]$benzene/carbon tetrachloride mixture, the reaction mixture was stirred for an additional three hours with the temperature maintained between 10-15° C. The reaction mixture was allowed to stand for 12 hours during which it reached room temperature. The excess carbon tetrachloride was removed from the reaction mixture with a Pasteur pipette. The reaction mixture was then cooled and 25 mL of deuterium oxide was slowly added. Following the addition of the deuterium oxide, the reaction mixture was extracted using diethyl-ether. The organic layer was transferred to a clean 250 mL round bottom flask and dried using anhydrous sodium sulfate. The organic layer was subsequently filtered and the solvents were removed under vacuum. The resulting product was purified using column flash chromatography (in a glass column of dimensions 6 cm X 75 cm packed with Silica gel 60; mesh 240-400; mobile phase is 97% n-hexane: 3% diethyl ether).
Fractions eluting from the column were collected, and those fractions shown to include the product (i.e., via thin layer chromatography) were pooled, dried with anhydrous sodium sulfate, and filtered. The solvent was removed under vacuum. The product was re-crystallized overnight from benzene (4°C). The yield of the vacuum dried $[^2\text{H}_{10}]$benzophenone was 60% based on the mass of $[^2\text{H}_6]$benzene. The product, $[^2\text{H}_{10}]$benzophenone, was converted to $[^2\text{H}_{10}]$benzhydrol, as described earlier for unlabeled benzhydrol (Wisegogle and Sonneborn, 1941) (Figure 2). The purified $[^2\text{H}_{10}]$benzophenone ($\sim 6 \text{ g}$) was added to a clean 250 mL round bottom flask along with 6 g of NaOH, 6 g Zinc dust, and 60 mL ethyl alcohol. A thermometer, a Teflon® coated magnetic stirrer, and a reflux condenser were attached to the round bottom flask. The mixture was gradually heated to 70°C, with stirring, and the temperature was held for 2 hours. The reaction mixture was filtered with suction, and the residue washed with two 3 mL aliquots of heated ethyl alcohol. The resulting filtrate was poured into ice cold 1.0 M HCl. The resulting crystals were filtered with suction, washed with ice cold distilled water, and dried under vacuum. This reaction was almost complete with a yield of $\sim 95\%$. The purified product, $[^2\text{H}_{10}]$benzhydrol, was used for the subsequent synthesis of stable isotope labeled compounds (i.e., $[^2\text{H}_{10}]$DPHM and $[^2\text{H}_{10}]$DPMA).
2.3.2. Synthesis of $[\text{^2H}_{10}]$DPHM HCl

The synthesis of $[\text{^2H}_{10}]$DPHM involved a one step reaction, as reported earlier for tritium labeled DPHM (Figure 3) (Blackburn and Ober, 1967). Into a clean three neck 250 mL round bottom flask 2.5 g of purified $[\text{^2H}_{10}]$benzhydrol, 1.5 mL dimethylaminoethanol, 2.7 g para-toluene sulfonic acid, 27 mL tetrachloroethane, and 41 mL toluene were added. A Teflon coated magnetic stirrer, a reflux condenser, a thermometer, and a water trap were added onto the reaction flask. The reaction was conducted under a nitrogen atmosphere. The reaction was
initiated by gradually heating the reaction mixture to 125°C with stirring. The reaction mixture was refluxed for 48 hours, cooled to 60°C, and diluted with 90 mL petroleum ether and 30 mL distilled water. The reaction mixture was transferred to a 500 mL separatory funnel. The bottom two aqueous layers were removed and transferred to a clean separatory funnel. The top organic phase was washed with two 20 mL aliquots of distilled water, and the washings were combined with the aqueous fraction. A 7.0 mL aliquot of 50% NaOH was added to the aqueous phase. Next, the aqueous phase was extracted with one 60 mL and two 30 mL portions of diethyl ether. The combined ether phase was washed with two 20 mL portions of water and then dried twice with 4 g portions of anhydrous magnesium sulfate. This mixture was filtered and the ether removed under vacuum. A 2 mL aliquot of isopropyl alcohol was added to the resulting oil, followed by an addition of 2.6 mL of isopropyl alcohol saturated with HCl (i.e., HCl gas was bubbled through the isopropyl alcohol). This was followed by the addition of 70 mL anhydrous diethyl ether. The mixture was allowed to stand at 4°C overnight to crystallize. The following morning the mixture was filtered and the resulting crystals dried under vacuum. The product was re-crystallized twice: once, using 2 mL of isopropyl alcohol and 60 mL anhydrous diethyl ether; the second time, using acetone and heat. The yield of this reaction was 50%.

![Diagram of synthesis of [2H10]DPHM](image)

Figure 3: Synthesis of [2H10]DPHM
2.3.2.1. Characterization of $[^2\text{H}_{10}]$DPHM

Identification of the $[^2\text{H}_{10}]$DPHM HCl was confirmed using $^1$H-NMR (Bruker AC-200 [200MHz], Dept. of Chemistry, University of British Columbia)[ $^1$H-NMR: (D$_2$O) $\delta$ 5.38 (s, 1H, CHO), 3.58 (t, 2H, OCH$_2$), 2.62 (t, 2H, CH$_2$), 2.28 (br s, 6H, N(CH$_3$)$_2$)] (Appendix 2). In addition, GC/LC/MS was also used in the identification of $[^2\text{H}_{10}]$DPHM. Characteristic fragments of $[^2\text{H}_{10}]$DPHM following GC-MS/EI were [$m/z$ (% abundance)]: $m/z$ 58(100), 73(59), 159(23), 173(48), 177(46), and 193(1). Direct flow injection HPLC-MS, which was conducted using a thermospray interface with the ion source in the positive ion mode, identified the ion $m/z$ 266 which corresponded to the [M+H]$^+$ fragment of $[^2\text{H}_{10}]$DPHM. In addition, a sharp melting point at 167°C (literature MP for DPHM is 166-170°C) was determined for $[^2\text{H}_{10}]$DPHM HCl using differential scanning calorimetry (DSC).

2.3.3. Synthesis of N-demethyl DPHM and N,N-didemethyl DPHM

N-demethyl diphenhydramine and N,N-didemethyl diphenhydramine were synthesized and purified as outlined above in section 2.3.2; however, rather than dimethylaminoethanol, methylaminoethanol and aminoethanol were used for the synthesis of N-demethyl DPHM and N,N-didemethyl DPHM, respectively.
2.3.3.1. Characterization of N-demethyl DPHM and N,N-didemethyl DPHM

The structures of N-demethyl DPHM and N,N-didemethyl DPHM were characterized using GC-MS (Scan). Characteristic ion fragments of N-demethyl DPHM were \([m/z \text{ (abundance)}]: m/z \text{ 59(100), 152(31), 165(79), 167(66), and 183(28)},\) and for N,N-didemethyl DPHM were \([m/z \text{ (abundance)}]: m/z \text{ 152(33), 165(73), 167(100), and 183(11)}\). In addition, H\(^1\)-NMR was also used to confirm the structures of N-demethyl DPHM HCl and N,N-didemethyl DPHM HCl (Bruker AC-200 [200MHz], Dept. of Chemistry, University of British Columbia; N-demethyl DPHM HCl \([^1H\text{-NMR: (D}_2\text{O)} \delta 7.40 \text{ (m,1OH, Aromatic), 5.53 (s, 1H, CHO), 3.70 (t, 2H, OCH}_2\text{), 3.25 (t, 2H, CH}_2\text{), 3.23 (s,3H, N(CH}_3\text{), 2.18 (s, 1H, NH)}\); N,N-didemethyl DPHM HCl \([^1H\text{-NMR: (D}_2\text{O)} \delta 7.40 \text{ (m,1OH, Aromatic), 5.58 (s, 1H, CHO), 3.70 (t, 2H, OCH}_2\text{), 3.25 (t, 2H, CH}_2\text{)}\]). The melting points of N-demethyl DPHM HCl and N,N-didemethyl DPHM HCl were 159 and 178°C, respectively, as determined by DSC.

2.3.4. Synthesis of DPMA and \([^2H_{10}]\text{DPMA}\)

The method for the synthesis of DPMA and \([^2H_{10}]\text{DPMA}\) was adapted from a method described earlier to synthesize only DPMA shown in figure 4 (Djerassi and Scholz, 1948). Into a clean 250 mL three neck round bottom flask with a Vigreux distillation column, dropping separatory funnel, a glass inlet tube, and a thermometer were added 0.7 g freshly cut sodium metal and 12 mL methanol. A gentle stream of dried nitrogen gas was passed through the glass inlet tube. This mixture was stirred, and gradually a mixture of 5.5 g of either \([^2H_{10}]\text{benzhydrol or benzhydrol in 24 mL of dry toluene was slowly added. The temperature of the reaction vessel was slowly increased to 140°C until a moderate rate of distillation was achieved. Aliquots of}
dry toluene were periodically added to keep the mixture liquid. Once the temperature in the Vigreux column had reached 90° C, the column was replaced with a reflux condenser. A 2.1 g portion of bromoacetic acid was added, followed by 20 mL of dry toluene. The reaction mixture was then refluxed for 2 hours. The reaction mixture was cooled, and 30 mL of distilled water was added. The reaction mixture was added to a clean 500 mL separatory funnel. The aqueous layer was alkalized (i.e., above pH 13.0) by the drop wise addition of 10 M NaOH. The toluene and aqueous layers were separated. The aqueous phase was washed with diethyl ether (2 aliquots of 25 mL), acidified by the drop-wise addition of 10 M HCl (i.e., pH 1), and extracted (3 aliquots of 20 mL) with diethyl ether. The ether layer was washed with two aliquots of 20 mL of distilled water or until the washings were neutral (i.e., pH ~7.0). The ether layer was then dried with anhydrous sodium sulfate and filtered. The ether was removed under vacuum. The resulting oil of either DPMA or [2H10]DPMA was purified using column flash chromatography (in a glass column of dimensions 6 cm X 75 cm packed with silica gel 60; mesh 240-400; mobile phase 50% diethyl ether: 48% hexane: 2% isopropyl alcohol) followed by re-crystallization with hexane and acetone. The yields of vacuum dried DPMA and [2H10]DPMA were 28% and 30% respectively.

2.3.4.1. Characterization of DPMA and [2H10]DPMA

The identification of DPMA and [2H10]DPMA was confirmed using 1H-NMR (Bruker AC-200 [200MZH], Department of Chemistry, University of British Columbia): [DPMA - 1H-NMR: (CDCl3); δ 4.20 (S, 2H, OCH2); 5.50 (S, 1H, CH); 7.30 (M, 10H, ArH)] and [2H10]DPMA - 1H-NMR: (CDCl3); δ 4.20 (S, 2H, OCH2). 5.50 (S, 1H, CH)]. In addition, gas chromatography-mass spectrometry (GC-MS), using both electron impact and negative chemical ionization, was
used in the identification of DPMA and $[^2\text{H}_{10}]$DPMA. Pure DPMA and $[^2\text{H}_{10}]$DPMA were dissolved in toluene, derivatized with MTBSTFA, and injected into the GC-MS (EI), which was used in the scanning mode. Characteristic fragments following GC-MS (EI) for DPMA were $[m/z \, (\% \, abundance)]: m/z \, 152 \, (20), \, 165(38), \, 167(100), \, 183(93), \, \text{and} \, 299(4)$, and for $[^2\text{H}_{10}]$DPMA were: 159(15), 173(19), 177(100), 193(95), and 309(4), respectively. Aliquots of DPMA and $[^2\text{H}_{10}]$DPMA dissolved in toluene were derivatized with PFBBBr. These PFBBBr derivatives of DPMA and $[^2\text{H}_{10}]$DPMA were subjected to GC-MS in the negative chemical ionization (NCI) mode with methane as the reagent gas. The total ion scan showed only one fragment for DPMA ($m/z \, 241$) and $[^2\text{H}_{10}]$DPMA ($m/z \, 251$), corresponding to the loss of the pentafluorobenzyl group from the molecular ion (i.e., [M-181]). Sharp melting points at 77 and 78°C were measured for DPMA and $[^2\text{H}_{10}]$DPMA, respectively, using DSC.

![Synthesis of DPMA and $[^2\text{H}_{10}]$DPMA](image)

Figure 4: Synthesis of DPMA and $[^2\text{H}_{10}]$DPMA
2.3.5. Purity Assessment of Synthesized Standards.

The purity of the DPHM HCl and the synthesized $[\text{H}_{10}]$DPHM HCl standards were assessed in the following fashion. Firstly, aqueous solutions of DPHM HCl or $[\text{H}_{10}]$DPHM HCl (100 µg/mL) were extracted with 2% isopropyl alcohol: 98% hexane with 0.05 M triethylamine (TEA). The organic phase was dried and reconstituted with 0.05 M TEA in toluene. An aliquot of the reconstituted samples (i.e., DPHM and $[\text{H}_{10}]$DPHM) was subjected to gas chromatography with nitrogen/phosphorus specific (NPD) detection, while the other aliquot of the reconstituted samples was assessed using GC-MS (scan mode). Only one chromatographic peak, other than those present in the blank, could be detected using GC-NPD and GC-MS/EI (total ion chromatogram) following injection of the prepared DPHM HCl and $[\text{H}_{10}]$DPHM HCl standards. Standard aqueous solutions of DPHM HCl and $[\text{H}_{10}]$DPHM HCl (100 µg/mL) were subjected to LC-MS via direct sample introduction into the LC-MS source through a thermospray interface. DPHM and $[\text{H}_{10}]$DPHM did not fragment extensively under these conditions, and therefore, only one ion was observed in each sample, which corresponded to the $[M+1]^+$ ions of DPHM, ($m/z$ 256) and $[\text{H}_{10}]$DPHM ($m/z$ 266). Thermal analysis was conducted using DSC. Data obtained showed only one sharp peak corresponding to a melting point of 167°C for both DPHM and $[\text{H}_{10}]$DPHM. The absence of other peaks during the thermal analysis also suggests the lack of any polymorphic forms, and/or solvates of the DPHM and $[\text{H}_{10}]$DPHM HCl standards.

The purity of N-demethyl DPHM and N,N-didemethyl DPHM was determined using GC-MS (Scan) and GC-NPD, as described above. The N-demethyl DPHM HCl and N,N-didemethyl were dissolved in 0.05 M TEA in toluene and injected directly into the GC. N-demethyl DPHM was deemed pure, since only one peak was detected in the chromatogram using both GC-MS
(Scan) and GC-NPD; however, the N,N-didemethyl DPHM HCl demonstrated a peak which eluted just following the metabolite peak. This peak was reduced by further re-crystallization, but was never eliminated (peak area counts with GC-NPD and GC-MS were <2% of the metabolite). Thermal analysis using DSC showed only one sharp peak for N-demethyl DPHM and N,N-didemethyl DPHM. The absence of other peaks during the thermal analysis also suggests the lack of any polymorphic forms, and/or solvates of the N-demethyl DPHM HCl and N,N-didemethyl DPHM HCl standards.

The purity of DPMA and [2H10]DPMA was assessed in the following fashion. DPMA and [2H10]DPMA were dissolved in toluene, an aliquot was removed and derivatized with MTBSTFA, and this aliquot was injected directly into the GC-MS/EI. The total ion chromatogram showed only the peaks corresponding to the tert-butyldimethylsilyl (TBDMS) derivatives of DPMA and [2H10]DPMA. No chromatographic peaks other than those present in the blank were detected. In addition, both DPMA and [2H10]DPMA were dissolved in distilled water. These samples were extracted with toluene from an acidified aqueous matrix. The toluene was dried under a gentle stream of nitrogen gas. The reconstituted residue was derivatized with either MTBSTFA (GC-MS/EI) or PFBBr (GC-MS/NCI). The total ion chromatograms showed only the chromatographic peaks corresponding to the compounds in question. Thermal analysis of DPMA and [2H10]DPMA conducted using DSC showed only one sharp peak corresponding to their respective melting points. The lack of other peaks during thermal analysis also suggests the lack of any polymorphic forms, and/or solvates and hydrates of the DPMA and [2H10]DPMA standards.
2.4. Analytical Method Development

2.4.1. Development of an Analysis Method for DPHM and $[^{2}\text{H}_{10}]$DPHM

2.4.1.1. Optimization of Mass Spectrometer Parameters

The fragment ions for selected ion monitoring (SIM) of both DPHM and $[^{2}\text{H}_{10}]$DPHM were chosen based on the retention of the stable isotope label and good abundance. The mass spectrometer was tuned using perfluorotributylamine (PFTBA), using a variety of tuning algorithms to optimize the sensitivity of the assay (i.e., programmed auto tune [m/z 69, 219, and 502], mid-mass tune [m/z 69, 219, and 265] and a manual tune [m/z 100, 131, and 219]). The mass spectrometer dwell time was set to allow 15 scans/chromatographic peak.

2.4.1.2. Optimization of Gas Chromatographic Parameters

Two capillary columns were tested for the quantitation of DPHM and $[^{2}\text{H}_{10}]$DPHM, namely, a HP Ultra-2, and a J&W DB-1701. The influence of injector purge times on analyte peak area counts was examined by varying purge times (i.e., 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 minutes) and repeated injections of a standard sample. The effect of the rate of column helium flow on column efficiency was also tested using a variety of column head pressures (7.5, 10, 12.5, 15, 17.5, and 20.0 P.S.I.). In addition, the effect of the injector temperature on the analyte peak area counts was measured (200, 225, 250, and 275 °C). Finally, the influence of the initial column temperature on half-height peak width was examined; a variety of initial column temperatures were assessed, namely, 70, 80, 90, 100, 110, 120, 130, 140, and 150 °C.
2.4.1.3. **Optimization of Extraction Procedure**

A variety of solvents were tested for the extraction efficiency and suitability (*i.e.*, ease of extraction and selectivity of the solvent) for the DPHM and [\(^2\text{H}_{10}\)]DPHM analysis method. Spiked plasma samples were basified with 1.0 M NaOH and extracted with either toluene, methylene chloride, hexane, or 98% hexane: 2% isopropyl alcohol. The peak area response and the chromatographic base lines (*i.e.*, lack of interfering chromatographic peaks) were used to choose the most appropriate solvent for extraction. In addition, a variety of hexane:isopropyl alcohol solvent compositions (*i.e.*, 0, 2, 5, 10, and 20% isopropyl alcohol) were examined in a similar fashion as described above. The influence of silanization of glassware, and the addition of triethylamine (TEA) at a variety of concentrations (*i.e.*, 0.025, 0.05, 0.10, and 0.20 M) on peak area counts were also examined. Once the appropriate extraction solvent system was determined, the effect of different extraction times (*i.e.*, 5, 10, 15, 20, 25, and 30 minutes) on the relative extraction recovery of DPHM and [\(^2\text{H}_{10}\)]DPHM was investigated.

2.4.1.4. **Gas Chromatograph - Mass Spectrometer Operating Conditions**

A 2.0 \(\mu\)L aliquot of prepared sample was injected through a Thermogreen\textsuperscript{®} LD-2 silicone rubber septa into the gas chromatograph split/splitless inlet equipped with a Pyrex\textsuperscript{®} glass inlet liner (78 mm X 4 mm i.d.) in the splitless mode (purge time 1.5 minutes). Chromatographic separation of DPHM, [\(^2\text{H}_{10}\)]DPHM, and orphenadrine from endogenous materials and the demethylated metabolites of DPHM and [\(^2\text{H}_{10}\)]DPHM was achieved using a 30 m J&W DB-
1701 0.25 mm i.d. (0.25 μ film thickness) capillary column. Column head pressure was set at
12.5 P.S.I. The gas chromatographic operating conditions were as follows: The injection port
temperature was held at 180º C. The initial oven temperature was maintained at 140º C for 1
minute, then the oven temperature was ramped at 30º C/minute to 200º C. The oven temperature
was again ramped at 17.5º C/minute from 200º C to 265º C, where it was held for 5.0 minutes.
The temperature program resulted in a total run time of 12.7 minutes. The transfer line
temperature was held at 280º C. The mass spectrometer (MS) was manually tuned with the
tuning reagent perfluorotributylamine (PFTBA) to ions m/z 100, 131, and 219. The GC-MS
operating in the electron impact ionization mode (voltage 70 eV) with selective ion monitoring
(EIF-SIM) was used to quantitate DPHM and [²H₁₀]DPHM by monitoring ions m/z 165 and 173,
respectively. The dwell time was set at 50 msec. for each ion being monitored, to ensure
adequate sampling of the chromatographic peak of interest (i.e., 15 scans/peak). The electron
multiplier voltage was programmed to +300 V relative to the tune value during the elution of the
compounds of interest. The voltage was programmed to reset to -1000 V relative to the tune
value at all other times to maximize the life span of the electron multiplier.

2.4.1.5. DPHM and [²H₁₀]DPHM Extraction Procedure

Samples were prepared for analysis by a single step liquid/liquid extraction procedure.
Aliquots of biological samples (0.1 - 1.0 mL) including maternal and fetal plasma, amniotic
fluid, tracheal fluid and urine were individually pipetted into clean borosilicate test tubes. The
samples were made up to volume (1.0 mL) with distilled water, internal standard (orphenadrine
200 ng), and 0.5 mL of 1 M NaOH were then added to the test tube along with 7.0 mL of solvent
(0.05 M TEA in 2% isopropyl alcohol: 98% hexane). The samples were capped and mixed for 20 minutes, cooled for 10 minutes at -5°C in a freezer in order to break any emulsion formed during mixing, and centrifuged for 10 minutes at 3000 x g. The organic phase was transferred to a clean test tube and dried in a water bath at 30°C under a gentle stream of nitrogen gas. The dried samples were reconstituted with 150 μL of 0.05 M TEA in toluene. The reconstituted samples were then transferred to clean borosilicate microvial inserts which were placed in standard borosilicate autosampler vials, from which a 2.0 μL aliquot was used for injection.

2.4.1.6. Preparation of a Calibration Curve

An eight point calibration curve was constructed from the aqueous standard solutions of DPHM and \([^{2}H_{10}]\)DPHM to yield concentrations of 2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 150.0 and 200.0 ng/mL using a prepared stock solution of 200.0 ng/mL of DPHM and \([^{2}H_{10}]\)DPHM. The blank plasma, fetal tracheal fluid, or amniotic fluid was added to the calibration curve samples. The samples were extracted and quantitated as described above. Weighted linear regression (weighting function = \(1/y^2\)) was performed between the mean drug response (DPHM or \([^{2}H_{10}]\)DPHM peak area/internal standard [orphenadrine] peak area) and the spiked drug concentrations of DPHM or \([^{2}H_{10}]\)DPHM.

2.4.1.7. Calculation of Extraction Recoveries of DPHM and \([^{2}H_{10}]\)DPHM

Extraction recoveries of both DPHM and \([^{2}H_{10}]\)DPHM were determined at low, moderate, and high concentrations (2.0, 50.0, and 200.0 ng/mL, respectively) from a variety of biological
matrices (maternal plasma, amniotic fluid, and tracheal fluid). Two groups of samples were used to assess extraction recovery, namely, a test group and a control group. Both groups of samples contained blank biological matrix (plasma, amniotic fluid, and fetal tracheal fluid) and internal standard. However, the samples from the test group were spiked with DPHM and $[^2\text{H}_{10}]$DPHM to yield final concentrations of 2.0, 50.0, and 200.0 ng/mL, whereas the samples in the control group were not spiked with DPHM and $[^2\text{H}_{10}]$DPHM at this point of the experiment. Following liquid-liquid extraction of both the test and control group samples, aliquots of DPHM and $[^2\text{H}_{10}]$DPHM standards, made up in methanol, were added to the control group samples to yield drug concentrations of 2.0, 50.0, and 200.0 ng/mL. Control and test samples were then dried, reconstituted, and chromatographed as described above. The concentrations of the test and control samples were determined from standard curves extracted from the corresponding biological matrices. The extraction recovery was calculated as the ratio of the measured concentration of the test samples over the measured concentration of the control samples at the low, medium, and high concentrations.

2.4.1.8. Sample Stability Assessment

Numerous studies were carried out in order to determine the stability of the samples during storage and analysis. The freezer stability of these samples was assessed by spiking blank maternal plasma with DPHM and $[^2\text{H}_{10}]$DPHM at known concentrations of 5.0, 25.0, and 100.0 ng/mL, and storing these samples at -20° C for up to 12 months. Samples were periodically removed (i.e., 0.5, 1, 2, 4, 6, 12 months) and the concentration of DPHM and $[^2\text{H}_{10}]$DPHM measured. These measured concentrations where then compared to the known concentrations.
The freeze-thaw stability of DPHM and $[^2\text{H}_{10}]$DPHM in plasma was assessed in the following manner. Blank plasma samples were spiked with 58 ng/mL of DPHM and $[^2\text{H}_{10}]$DPHM. These samples were frozen at -20°C and thawed at 22°C on the bench-top. This cycle was continued for a total of three cycles. On the final cycle, the samples were frozen at -20°C and stored until analysis. The bench-top stability of DPHM and $[^2\text{H}_{10}]$DPHM in a plasma matrix was determined in the following fashion. Blank plasma was spiked with DPHM and $[^2\text{H}_{10}]$DPHM to yield a concentration of 58 ng/mL. The samples were left on the bench-top at 22°C for various periods of time (i.e., 0, 1, 2, 4, 6, 12, and 24 hours). The samples were immediately frozen at -20°C and stored frozen until the time of analysis. A stability study of the extracted and derivatized samples stored on the autosampler tray of the GC-MS was conducted. Prepared samples at concentrations of 2.0 and 200.0 ng/mL of DPHM and $[^2\text{H}_{10}]$DPHM were extracted and analyzed. These samples were repeatedly injected at 24 hour intervals for a total of 72 hours (i.e., 0, 24, 48, and 72 hours).

2.4.1.9. Method Validation

Intra-day variability was determined by quantitating six replicates at concentrations of 2.0, 20.0, 100.0, and 200.0 ng/mL on one experimental day. Inter-day variability was determined by quantitating one sample in duplicate at concentrations of 2.0, 20.0, 100.0, and 200.0 ng/mL on six different experimental days.

The GC-MS method for the quantitation of DPHM and $[^2\text{H}_{10}]$DPHM was independently cross-validated by quantitating samples of DPHM and $[^2\text{H}_{10}]$DPHM individually by the GC-MS method developed, and by a published capillary gas chromatographic analysis for the
quantitation of DPHM using a GC-NPD (Yoo et al., 1986). In order to be able to quantitate both DPHM and $[^{2}\text{H}_{10}]$DPHM using GC-NPD, calibration curves and samples were prepared individually, that is, DPHM and $[^{2}\text{H}_{10}]$DPHM were not present together in the same sample (only for cross validation samples). This was done because the GC-NPD method could not differentiate between DPHM and $[^{2}\text{H}_{10}]$DPHM, if they were present in the same sample.

2.4.2. Development of an Analysis Method for DPMA and $[^{2}\text{H}_{10}]$DPMA

2.4.2.1. Optimization of Mass Spectrometer Conditions

The mass spectrometer conditions were optimized as described above (2.4.1.1.)

2.4.2.2. Optimization of Gas Chromatograph Conditions

Two capillary columns were tested for use in the quantitation of DPMA and $[^{2}\text{H}_{10}]$DPMA (i.e., HP Ultra-2 and J&W DB-1701). The effect of the rate of column helium flow on column efficiency was also tested using a variety of column head pressures (7.5, 10, 12.5, and 15 P.S.I.). In addition, the effect of the injector temperature on the analyte peak area counts was measured (250, 260, 270, 280, 290, 300°C). Finally, the influence of the initial column temperature on peak width was examined (150, 160, 170, 180, 190, 200°C).
2.4.2.3. **Optimization of Extraction**

Two solvents were investigated for the extraction of DPMA and $[\text{^{2}H}_{10}]$DPMA from ovine plasma and urine matrices. The peak area counts and the quality of the chromatography were assessed following drug extraction with ethyl acetate and toluene from acidified urine and plasma matrices.

2.4.2.4. **Optimization of Derivatization**

Two derivatization techniques were examined during the development of the DPMA and $[\text{^{2}H}_{10}]$DPMA analysis method. Pentfluorobenzyl or tert-butylidimethylsilyl derivatives were formed using the reagents pentfluorobenzylbromide (PFBBr) and N-methyl-N-(tert-butylidimethylsilyl)trifluoroacetamide (MTBSTFA). Samples of DPMA and $[\text{^{2}H}_{10}]$DPMA were extracted from acidified (pH ~1.5) biological matrices (plasma and urine). The toluene layer, removed following the extraction, was dried and the residues derivatized. In the case of the PFB derivatives, 200 µL of a solution containing 1 part PFBBr and 100 parts acetone, and a ~0.5 g aliquot of anhydrous sodium sulfate were added to the dried residue. These samples were capped and incubated at 60°C for 1 hour. Residual PFBBr was neutralized with the addition of 0.5 mL of distilled water and vortex mixed for ~30 seconds. The organic layer was removed and dried under a gentle stream of nitrogen gas at 40°C and reconstituted with 200 µL toluene. To form the TBDMS derivatives the residue resulting from the evaporation of the organic extraction solvent was reconstituted with 200 µL toluene and 50 µL of MTBSTFA. This mixture was incubated for 60 minutes at 60°C. The effect of various volumes of MTBSTFA (*i.e.*, 5, 10, 25, 50 and 100 µL) on DPMA, $[\text{^{2}H}_{10}]$DPMA, and DPAA peak areas was also assessed. In addition,
incubation times for the MTBSTFA derivatization procedure were also optimized (i.e., times tested 30, 60, 90, 120 minutes).

2.4.2.5. Gas Chromatograph-Mass Spectrometer Operating Conditions

A 1.0 μL aliquot of prepared sample was injected through a Thermogreen® LD-2 silicone rubber septum into the split/splitless capillary inlet equipped with a Pyrex® glass inlet liner (78 mm X 4 mm i.d.) operated in the splitless injection mode with a purge time of 1.5 minutes. Chromatographic separation of the analytes ([2H10]DPMA, DPMA, and DPAA) from endogenous materials was achieved using a 25 meter HP Ultra-2 0.2 mm i.d. (0.33 μ film thickness) capillary column. Column head pressure was optimized at 15 P.S.I. (corresponding to 0.6 mL/minute at the initial temperature). The gas chromatographic system operating conditions were as follows: The injection port temperature was held at 280° C. The initial oven temperature was maintained at 125° C for 1 minute, the oven temperature was ramped at 12.5° C/minute to 280° C, where it was held for 4.0 minutes. The temperature program resulted in a total run time of 17.4 minutes. The transfer line temperature was held at 285° C. The MS was manually tuned using the tuning reagent perfluorotributylamine (PFTBA) to ions m/z 100, 131, and 219. The GC-MS operating in the electron impact ionization mode (voltage 70 eV) with selective ion monitoring (EI-SIM) was used to quantitate DPAA, DPMA, and [2H10]DPMA by monitoring ions m/z 165, 183 and 177, respectively. The dwell time was set at 125 msec for each ion being monitored to ensure adequate sampling of the chromatographic peak of interest (i.e., 15 Scans/chromatographic peak). The electron multiplier voltage was programmed to + 200 V relative to the tune value during the elution of the compounds of interest. The voltage was
programmed to reset to -1000 V relative to the tune value at all other times to maximize the life span of the electron multiplier.

2.4.2.6. DPMA and [²H₁₀]DPMA Extraction Procedure

Samples were prepared for analysis using a single-step liquid-liquid extraction. Aliquots of biological samples (0.10-1.00 mL plasma and 0.05-1.00 mL urine) were pipetted into clean borosilicate test tubes. The samples were made up to a volume of 1.0 mL with distilled water. A 200 µL aliquot of internal standard (diphenylacetic acid: DPAA 2.0 µg/mL), 400 µL of 1.0 M HCl, and 5.0 mL of toluene were added to the biological sample. The test tubes were capped with PTFE-lined lids and mixed for 20 minutes, cooled for 10 minutes at -20°C in a freezer (to break any emulsion formed during mixing), and centrifuged at 3000 x g. The organic layer was transferred to clean test tubes and evaporated to dryness in a water bath at 40°C under a gentle stream of nitrogen gas. The dried samples were reconstituted with 200 µL dry toluene (toluene stored on anhydrous sodium sulfate), and 25 µL of the derivatizing reagent MTBSTFA was added. The tubes were capped, mixed for one minute on a vortex mixer, and incubated at 60°C for one hour. After the samples cooled, the derivatized mixture was transferred to clean borosilicate microvial inserts (placed in standard borosilicate autosampler vials) from which a 1.0 µL aliquot was used for injection.

2.4.2.7. Preparation of a Calibration Curve

A seven-point calibration curve was constructed from aqueous standard solutions of DPMA and [²H₁₀]DPMA to provide concentrations of 2.5, 5.0, 10.0, 25.0, 50.0, 125.0, and 250.0
ng/mL with 400 ng of DPAA added as the internal standard. Aliquots of blank plasma or urine were added to the calibration curve samples. The samples were extracted and quantitated as described above. Weighted linear regression (weighting function = 1/y²) was performed between the drug response (DPMA or [²H₁₀]DPMA peak area/internal standard (DPAA) peak area) and the spiked drug concentrations of DPMA or [²H₁₀]DPMA.

2.4.2.8. Calculation of Extraction Recovery of DPMA and [²H₁₀]DPMA

Extraction recoveries of DPMA and [²H₁₀]DPMA from plasma and urine were both determined at low, moderate, and high concentrations (5.0, 50.0, and 500.0 ng/mL). Extraction recoveries of DPMA and [²H₁₀]DPMA were determined as outlined in section 2.4.1.6. for DPHM and [²H₁₀]DPHM.

2.4.2.9. Sample Stability Assessment

Numerous studies were carried out in order to determine the stability of the samples during storage and analysis. The freezer stability of these samples was determined by spiking blank plasma with DPMA and [²H₁₀]DPMA at a known concentration of 100.0 ng/mL. These samples were stored at -20° C, removed at specific intervals (1, 2, 4, and 6 months), and analyzed. The freeze-thaw stability, bench-top stability, and injector stability of DPMA and [²H₁₀]DPMA in plasma samples were assessed as described in section 2.4.1.8. for DPHM and [²H₁₀]DPHM. Because DPMA and [²H₁₀]DPMA appeared to be sensitive to acid, the stability of the samples in an acidified matrix (i.e., acidified to the same degree as during the extraction procedure) was
examined. Briefly, spiked samples in a distilled water, plasma, and urine matrix were acidified with 400 μL of 1.0 M HCl. These samples were vortex mixed for one minute and left on the bench-top for the following periods of time: 0, 0.5, 1.0, 2.0, 4.0, 6.0, and 21.0 hours. Following the desired incubation time, the internal standard was added and the samples extracted as described above. The degradation half-lives of DPMA and [\(^{2}\text{H}_{10}\)]DPMA were determined in the biological matrices examined.

2.4.2.10. Method Validation

Intra-day variability was determined by quantitating four replicates at concentrations of 2.5, 25.0, 125.0, and 250.0 ng/mL using the GC-MS method reported above for the quantitation of DPMA and [\(^{2}\text{H}_{10}\)]DPMA on one experimental day. Inter-day variability was determined by quantitating one sample in duplicate at concentrations of 2.5, 25.0, 125.0, and 250.0 ng/mL on four different experimental days.

2.5. Standard Procedures for Animal Experiments

2.5.1. Animal Handling

Both pregnant and non-pregnant ewes of Suffolk, Finn, and Dorset mixed breed were used in these studies. The animals were brought into the animal unit at the Children’s Variety Research Center at least 1 week prior to surgery, and kept in groups of two or more in large pens in full view of one another. The animals received a standard diet and free access to water.
Ethical approval for the studies was obtained from the Animal Care Committee of the University of British Columbia, and the procedures used were in accordance to the guidelines of the Canadian Council of Animal Care.

2.5.2. Surgical Preparation for Chronic Experimentation

2.5.2.1. Non-pregnant Sheep - Surgical Preparation

Non-pregnant Dorset, Suffolk, and Finn mixed breed ewes were used in these studies. The ewes were allowed free access to water; however, food was withheld for approximately 18 hours prior to surgery. Aseptic techniques were employed throughout the surgical procedure. Approximately 20 minutes following intravenous atropine (6.0 mg) administration to control salivation, anesthesia was induced with intravenous sodium pentothal (1.0-1.5 g). The animals were intubated with an endotracheal tube and anesthesia was maintained through the ventilation of the animal (12 cycles/minute) with a mixture of halothane (1-2%), nitrous oxide (70%) and balance oxygen. An intravenous bolus injection of 500 mg ampicillin via the jugular vein was followed by the intravenous drip administration of a solution of 5% dextrose (500 mL) containing 80 mg gentamicin, at a rate of 5.0 - 10.0 mL/minute. The ewe’s abdomen, flank, and groin were shaved, and the surgical areas sterilized with 10% povidone-iodine topical solution, while other areas were covered with sterile sheets and drapes. Subcutaneous injections of 2% lidocaine were made along the site of the incision. Prior to implantation, all catheters were filled with heparinized saline (12 U/mL). Silicone rubber catheters (1.02 mm i.d. 2.15 mm o.d.) were implanted in the femoral artery and vein. An abdominal incision to the right of the umbilicus
was made to gain access to the gall bladder, where a catheter was placed to allow for bile collection. In addition, a (0.64 mm i.d. 1.19 mm o.d.) catheter was implanted in a small branch of the mesenteric vein, with the catheter tip lying in the main mesenteric vein in the direction of the hepatic portal vein of the ewe. The catheters were exteriorized, tunneled subcutaneously through an incision on the flank, and secured in a denim pouch. The catheters were filled with fresh heparinized sterile saline and capped when not in use. Tracheal catheters for N₂ gas infusion were implanted in order to induce hypoxemia. The neck of the ewe was shaved, and sterilized with 10% povidone-iodine topical solution. A small incision was made near the midline of the neck 4-5 cm below the larynx. The trachea was exposed. The endotracheal tube was deflated and a small hole was cut through the trachea between the cartilage rings of the trachea. A polyvinyl tube (5 mm i.d.) was rapidly inserted and glued into place with Tissue Glue® (3M, Minneapolis, MN, U.S.A.). The endotracheal tube was then re-inflated. The tube was anchored to the surrounding tissue with 2-0 silk and the incision closed. Ampicillin (500 mg) and gentamicin (40 mg) were administered prophylactically IM on the day of surgery and the following four days. The ewes were allowed to recover for at least five days before they were used in experiments. Just prior to each experiment, a size 16 fr. Foley® catheter was inserted into the bladder via the urethra for total urine collection.

2.5.2.2. Pregnant Sheep - Surgical Preparation

Time dated pregnant Dorset, Suffolk, and Finn mixed breed ewes were operated on between 115 and 125 days of gestation (term = 145 days). Ewes were allowed free access to water, but food was withheld for approximately 18 hours prior to surgery. Aseptic techniques
were employed throughout the surgical procedure. Following intravenous atropine (6.0 mg) administration to control salivation, anesthesia was induced with intravenous sodium pentothal (1.0-1.5 g). The animals were intubated with an endotracheal tube and anesthesia was maintained through the ventilation (12 cycles/minute) of the animals with a mixture of halothane (1-2%), nitrous oxide (70%), and balance oxygen. An intravenous bolus injection of 500 mg ampicillin was given via the jugular vein, followed by an intravenous drip of 5% dextrose solution (500 mL) containing 80 mg of gentamicin, at a rate of 5.0 - 10.0 mL/minute. The ewe’s abdomen, flank, and groin were shaved, and the surgical areas sterilized with 10% povidone-iodine topical solution, while other areas were covered with sterile sheets and drapes. Subcutaneous injections of 2% lidocaine were given along the site of the incision. A midline abdominal incision was made in the ewe and the uterus identified. Access to the head of the fetus was gained through an incision of the uterine wall in an area devoid of placental cotyledons and major blood vessels. With a small incision (1 cm) the fetal trachea was exposed. Again, all catheters were filled with heparinized (12 U/mL) normal saline prior to implantation. The silicone rubber catheter (1.02 mm i.d. 2.16 mm o.d.) was inserted into the trachea through a small incision through the fetal skin 1-2 cm below the larynx (See Appendix 3). The catheter was inserted through a small hole in between two rings of cartilage, and was then advanced 4-5 cm into the trachea towards the fetal lung. The tracheal catheter did not obstruct lung fluid efflux from the airway into the pharynx. The catheter was anchored onto the skin overlying the incision with a piece of 3-0 silk attached to the catheter. A drop of Tissue Glue® was applied on the catheter’s point of entry to the trachea. The fetal carotid artery was then dissected free from other tissue and three pieces of 3-0 silk were passed underneath the vessel. After the vessel was tied off with the distal silk, the proximal portion of the vessel was temporarily constricted. Then,
a partial cut was made on the vessel between the middle and the distal sutures. Approximately 3-4 cm of the silicone rubber catheter (0.64 mm i.d. 1.19 mm o.d.) was threaded through the cut on the vessel towards the ascending aorta (See Appendix 3). The catheter was secured to the vessel with all three silk sutures, and a drop of Tissue Glue® was applied to the site of entry. The catheter was anchored to the adjacent muscle of either side of the incision with the sutures. The tracheal incision was then closed with 2-0 silk. To implant the electro-cortical and electro-ocular electrodes, an incision was made laterally across the entire width of the fetal skull. The skin was peeled back and the fetal skull was exposed. Small holes were drilled (0.25 mm) biparietally through the skull. Electrodes consisting of multistranded stainless steel wire, insulated with Teflon® except at the tip and threaded through a ~ 5 mm small plastic disc, were inserted through these holes onto the dura. To seal the hole, the plastic disc was glued to the skull using Tissue Glue®. A third electrode wire (ground wire) was then sutured to the surrounding skin tissue. The incision was sutured back together using 2-0 silk. To insert the electro-ocular electrodes, small incisions were made (~1 cm) above the orbital ridge on either side of the fetal head. The electrode wires were driven through the orbital ridge of the zygomatic bone, with the uninsulated portion of the wire sutured through the bone and glued with Tissue Glue®. Next, an amniotic catheter (1.02 mm i.d. 2.16 mm o.d.) was placed in the amniotic fluid and anchored to the neck of the fetus. The fetus was then placed back into the uterus, after which the uterus was closed with a continuous 2-0 gut chromic suture, and then oversewn. Next, a second uterine incision was made to expose the fetal hindquarters. An incision (2 cm) was made above the femoral arterial pulse in the groin. The vessel was prepared for catheterization as discussed above for the carotid artery. Approximately 5-6 cm of the catheter was threaded through the cut on the femoral artery towards the descending aorta. Both right and left fetal
femoral arteries were catheterized in a similar fashion. Following catheterization, the catheters were secured and anchored as described above for the carotid artery. In a similar manner, silicone rubber catheters were placed into the right and left fetal lateral tarsal veins. Approximately 11-12 cm of the catheters were threaded into the vessel in order to reach the inferior vena cava. A Transonic® flow probe was placed around the common umbilical artery to measure fetal umbilical flow. To implant the flow probe, a flank incision (3-4 cm) was made 2-3 cm lateral to the spine, from just central to the kidney to the iliac crest. The common umbilical artery was approached retroperitoneally and dissected clear of other tissues. A transit-time blood flow transducer, size 4SB was then placed around the artery, and the flow transducer cable was anchored to surrounding tissue. In addition, the ipsilateral internal iliac artery was ligated to reduce the non-placental components of the common umbilical arterial flow. The incision was closed in layers, and finally the skin was sutured with 2-0 silk. To catheterize the fetal bladder, a suprapubic incision was made. The fetal bladder was exposed and a purse string suture of a diameter of 2 cm was made using 3-0 silk. A small cut was made into the bladder inside the purse string and the catheter was inserted. The purse string suture was tied. An additional 2-0 suture was wrapped around the catheter and the fetal bladder tissue. The catheter was anchored to both the fetal abdominal muscle wall and the abdominal skin. The incision was closed in layers and finally the skin was sutured using 3-0 silk. To catheterize the common umbilical vein, a small incision was made in the umbilicus overlying one of the two umbilical veins. The tissue surrounding the umbilical vein was carefully dissected away from the surface of the vessel. Two sutures (5-0 silk) were placed, in parallel, through the vessel wall at right angles to the long axis and separated by 1 mm. Using an 18-gauge needle, a hole was made in the vessel wall between the sutures, and a silicone catheter (0.51 mm i.d. 0.94 mm o.d.) was inserted ~2 cm, so that the
tip of the catheter lay in the intra-abdominal common umbilical vein. The sutures were tied in a "figure 8" fashion around the catheter and a drop of Tissue Glue\textsuperscript{®} was applied. The catheter was anchored to the skin overlying the umbilicus and to skin of the fetal abdomen. The incision was then closed with 3-0 silk. Two additional amniotic catheters were anchored to the fetal abdominal skin. The fetus was then gently returned to the uterus. Amniotic fluid lost during surgery was replaced with irrigation saline (Tranvenol Canada Inc., Mississauga, Ont.), after which the uterine incision was closed with a continuous 2.0 gut chromic suture, and then oversewn. The catheters were flushed with heparinized normal saline (12 U/mL), tunneled subcutaneously, and exited through a small incision on the maternal right flank. The ewe’s midline abdominal incision was closed in layers and the flank incision was sewn up as well. Finally, the maternal femoral artery and vein were catheterized (1.02 mm i.d. 2.15 mm o.d.). All catheters were capped and stored in a denim pouch which was secured in place with two adhesive bandages on the right flank. The ewe’s abdomen was then wrapped with elastic crepe bandages. All vascular catheters were flushed daily with 2 mL of heparinized normal saline. In the case of the umbilical venous catheter, an additional 0.5 mL of heparin (1000 U/mL) was used to prevent clot formation. Ampicillin (500 mg) and gentamicin (80 mg) were administered intramuscularly to the ewe on the day of surgery and for the first four days following surgery, while ampicillin 500 mg and gentamicin (20 mg) were administered IV to the fetus at the time of surgery. Ampicillin (500 mg) and gentamicin (20 mg) were also administered into the amniotic cavity on a daily basis until delivery. The ewes were moved into holding pens in the company of other sheep and were allowed to recover for at least 5 days following surgery. On the day of the experiment, a 16 fr. Foley\textsuperscript{®} catheter was inserted into the maternal bladder via the urethra and attached to a polyvinyl bag for cumulative urine collections.
2.5.3. Chronic Monitoring of Animals

2.5.3.1. Amniotic, Tracheal, and Vascular Pressures, Heart rate, Blood Flow, ECoG and EOG.

Prior and during experiments, amniotic, tracheal pressure and fetal and maternal arterial pressures were continuously monitored using disposable DTX transducers. Fetal and maternal heart rates were measured from the arterial pulse or from arterial blood flow by means of cardiotachometers (Model 9857). Electrocortical and electro-ocular signals were recorded using a type 9806A AC/DC coupler. Umbilical flow was measured with a transit time flowmeter (Model T201, Transonic Systems, Ithica, NY, U.S.A.). All variables were recorded using a Beckman R-711 polygraph recorder in conjunction with a computerized data acquisition system (Kwan, 1989). The sampling rate was 2.5 Hz. At the end of each minute, the measurements were averaged, fetal arterial pressure corrected for amniotic pressure, and the values displayed on the computer monitor. Every 30 minutes, the minute average measurements were automatically transferred to floppy diskettes for subsequent analysis.

2.5.3.2. Fetal Urine Measurements

Fetal urine collections were made using a computer controlled roller pump assembly developed in our laboratory. This setup incorporated a disposable DTX transducer connected to a gravity fed urine reservoir (a sterile 10 mL syringe barrel) to which the fetal bladder catheter was connected. As the pressure in the reservoir increased above a preset trigger pressure (usually
3 mm Hg), the computer activated the roller pump (DIAS, Ex154, DIAS Inc. Kalamazoo, MI, U.S.A.) which would pump a calibrated volume of urine from the reservoir back to the amniotic cavity (via the amniotic catheter) during control periods, or into sterile sample collection syringes during experiments. The cumulative volume pumped per minute, which equals fetal urine production per minute, was recorded and stored on diskette.

2.5.3.3. Blood Gas Analysis

Blood pH, Po2, and Pco2 were measured using an IL 1306 pH/Blood gas analyzer (Allied Instrumentation Laboratory, Milan, Italy), with temperature correction to 39°C for maternal blood and 39.5°C for fetal samples. Blood oxygen saturation and hemoglobin content were measured using a Hemoximeter (Radiometer, Copenhagen, Denmark).

2.5.3.4. Glucose and Lactate measurements

Glucose and lactate measurements were made using a 2300 STAT plus glucose/lactate analyzer.

2.5.4. Dosage Preparation

DPHM HCl (Sigma Chemical Co. St. Louis, MO), and [2H10]DPHM HCl were weighed to obtain the correct dose for administration. The weighed doses were dissolved in sterile 0.9% sodium chloride for injection and then filtered through a 0.22 μ nylon syringe filter into a capped
empty clean sterile injection vial. All doses were made on the morning of the experiment, and were not stored for more than 6 hours at 4° C.

2.6. Experimental Protocols

2.6.1. Adult Non-Pregnant Studies

A 100 mg IV bolus of DPHM (over one minute) was administered via the femoral vein to five surgically prepared non-pregnant sheep. Serial arterial blood samples were drawn at -5, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180, 240, 300, 360, 480, 600 and 720 minutes. Urine was collected at -5, 15, 30, 45, 60, 90, 120, 150, 180, 240, 360, 480, 600, and 720 minutes (in one animal, collection was extended to 24 hours). The urine pH and volume was measured and recorded. In two of the animals, bile was collected over 10 minute intervals at 5, 15, 30, 45, 60, 90, 120, 150, 180, 240, 360, 480, 600, and 720 minutes. The concentration of DPHM in these biological samples was measured using a previously published GC-NPD method (Yoo et al., 1986).

2.6.2. Adult Isotope Effect Studies

In the adult isotope effect control experiments equimolar amounts of DPHM and \[^2\text{H}_{10}\text{DPHM}\] equivalent to approximately 50 mg each of DPHM and \[^2\text{H}_{10}\text{DPHM}\] free base were simultaneously administered as an intravenous bolus via the maternal femoral vein over one minute. Serial maternal femoral arterial blood samples (5 mL) were collected at -5, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 105, 120, 150, 180, 240, 310, 480, 600, and 720 minutes. Urine was collected at -5, 15, 30, 45, 60, 90, 120, 150, 180, 240, 360, 480, 600, 720, and 1440 minutes.
The urine pH and volume were measured and recorded. The concentrations of DPHM and $[^{2}\text{H}_{10}]$DPHM in the biological samples collected were measured using the developed GC-MS method described above (2.4.1).

2.6.3. Fetal Isotope Effect Studies

2.6.3.1. Fetal Bolus

The fetal control experiments involved the simultaneous administration of DPHM and $[^{2}\text{H}_{10}]$DPHM equivalent to approximately 5.0 mg each of DPHM and $[^{2}\text{H}_{10}]$DPHM free base via the fetal lateral tarsal vein. Fetal femoral arterial blood, amniotic fluid, and fetal tracheal fluid samples were collected at -5, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 120, 150, 180, 210, 240, 300, 360, 480, 600, and 720 minutes. The concentrations of DPHM and $[^{2}\text{H}_{10}]$DPHM in these biological samples were measured using the GC-MS method described above (2.4.1).

2.6.3.2. Fetal Infusion

Two control experiments were conducted to verify the absence of an isotope effect between the disposition of $[^{2}\text{H}_{10}]$DPHM and the metabolite $[^{2}\text{H}_{10}]$DPMA, compared to DPHM and DPMA. Equimolar doses of DPHM and $[^{2}\text{H}_{10}]$DPHM were simultaneously administered via the fetal lateral tarsal vein as a 2.0 mg loading dose followed immediately by a 90 minute infusion with an infusion rate of 60 µg/minute each of DPHM and $[^{2}\text{H}_{10}]$DPHM. Serial samples were collected from the fetal femoral and carotid arteries and maternal femoral arteries at -5, 5, 15, 30,
45, 60, 75, and 90 minutes. Amniotic fluid and fetal urine samples were also collected at -5, 30, 60, and 90 minutes. The concentrations of DPHM and [\textsuperscript{2}H\textsubscript{10}]DPHM in the collected biological samples were measured using the GC-MS method described above (2.4.1.). In addition, the concentrations of DPMA and [\textsuperscript{2}H\textsubscript{10}]DPMA were measured in these samples using the GC-MS method described above (2.4.2.).

2.6.4. Adult First-Pass Metabolism

2.6.4.1. Adult First-Pass Metabolism During Normoxia

In the adult first-pass metabolism study equimolar amounts of DPHM and [\textsuperscript{2}H\textsubscript{10}]DPHM (approximately equivalent to 50 mg each of DPHM and [\textsuperscript{2}H\textsubscript{10}]DPHM) were administered simultaneously via different routes (i.e., femoral vein - control and mesenteric vein - test), over one minute. For example, DPHM would be administered via the mesenteric vein simultaneously with [\textsuperscript{2}H\textsubscript{10}]DPHM via the femoral vein. It should be noted that in each animal the route of administration of DPHM and [\textsuperscript{2}H\textsubscript{10}]DPHM was alternated so that [\textsuperscript{2}H\textsubscript{10}]DPHM and DPHM were not administered via the same routes of administration in every animal. This procedure was incorporated into the experimental protocol to avoid any subtle isotope effects not detected by the isotope effect studies in non-pregnant and fetal sheep. Serial maternal femoral arterial blood samples (5 mL) were collected at -5, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 105, 120, 150, 180, 240, 360, 480, 600, and 720 minutes. The concentrations of DPHM and [\textsuperscript{2}H\textsubscript{10}]DPHM were measured in these samples using the GC-MS method described above (2.4.1.).
2.6.4.2. Adult First-Pass Metabolism During Mild Hypoxemia

The experimental protocol for the hypoxemia first-pass experiments was similar to that for the normoxic experiments (2.6.4.1). However, 15 minutes prior to the start of drug administration and for the first 6 hours following drug administration, nitrogen gas was infused at 7 L/minute via the tracheal catheter to reduce the inspired $O_2$ concentration. The maternal arterial blood gas status was periodically monitored. When the Po$_2$ rose above 70 mm Hg, the nitrogen flow was increased up to 12 L/minute. Six hours following the bolus dosing, the nitrogen gas flow was turned off, and the oxygen status rapidly returned to baseline. The concentrations of DPHM and [$^{2}$H$_{10}$]DPHM were measured in these samples using the GC-MS method described above (2.4.1.).

2.6.5. Fetal Hepatic First-Pass Metabolism

2.6.5.1. Fetal Umbilical Venous Bolus

The fetal umbilical first-pass experiments involved the simultaneous administration of DPHM and [$^{2}$H$_{10}$]DPHM (equivalent approximately to 5.0 mg (E#499, E#1143, E#543, and E#975) and 2.5 mg (E#208 and E#989) each of DPHM and [$^{2}$H$_{10}$]DPHM free base via different routes (e.g., DPHM administered via the umbilical vein and [$^{2}$H$_{10}$]DPHM administered via the fetal lateral tarsal vein). An additional precaution of alternating the route of administration of DPHM (i.e., tarsal or umbilical venous) and [$^{2}$H$_{10}$]DPHM (i.e., umbilical or tarsal venous) was incorporated into the experimental protocol to avoid any subtle isotope effects not detected by the isotope effect studies in non-pregnant and fetal sheep. In all cases, the injection of drug was
given over one minute. Serial samples of fetal femoral (2 mL) and carotid (2 mL) arterial blood, fetal tracheal fluid (2 mL) and amniotic fluid (5 mL) were collected at -5, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 105, 120, 150, 180, 210, 240, 300, 360, 480, 600, and 720 minutes. The concentrations of DPHM and $[^{2}\text{H}\text{I}]\text{DPHM}$ were measured in these samples using the GC-MS method described above (2.4.1.).

### 2.6.5.2. Fetal Umbilical Venous Infusion

The simultaneous fetal umbilical and tarsal venous infusion experiments involved the administration of an IV bolus loading dose of 2.0 mg of DPHM and $[^{2}\text{H}\text{I}]\text{DPHM}$ immediately followed by a 90 minute infusion of approximately 60 µg/minute of DPHM and $[^{2}\text{H}\text{I}]\text{DPHM}$ via the umbilical and tarsal veins, respectively. Again, the routes of administration of DPHM and $[^{2}\text{H}\text{I}]\text{DPHM}$ were alternated in subsequent animals. Serial blood samples were drawn from the fetal femoral and carotid arterial, and the fetal tracheal and amniotic catheters at -5, 5, 15, 30, 45, 60, 75, and 90 minutes. In these studies, paired femoral and carotid arterial blood gas samples were taken and measured. The concentrations of DPHM and $[^{2}\text{H}\text{I}]\text{DPHM}$ were measured in these samples using the GC-MS method described above (2.4.1.).

### 2.6.6. Paired Fetal/Maternal Infusion

Prior to drug administration, 10 sterile (20 mL) heparinized syringes were used to draw up 15 mL of drug free maternal arterial blood. These samples were stored refrigerated (4°C) until required for fetal blood replacement (syringes were removed from the refrigerator to allow blood
to warm up to room temperature just prior to infusion). Fetal blood withdrawn by sampling was slowly replaced following sample collection with drug free maternal blood at 1.0 hour intervals during the infusion and at 2.0, 6.0, 12.0, and 24 hours post infusion via a 10 minute infusion to the fetal femoral artery. DPHM was administered as a 20 mg IV loading dose over a 1.0 minute interval immediately followed by an infusion of 670 µg/minute via the maternal femoral vein. Simultaneously a fetal 5.0 mg IV loading dose of [2H10]DPHM was given over a 1.0 minute interval immediately followed by an infusion of [2H10]DPHM at 170 µg/minute, given via the fetal lateral tarsal vein. Simultaneous fetal and maternal blood samples were collected from the fetal femoral and carotid arteries, umbilical vein, and maternal femoral artery. Blood samples (fetal [2.5 mL] and maternal [5.0 mL] arterial samples) were collected at -5, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, and 360 minutes during the infusion and at 30, 60, 120, 180, 240, 360, 480, 720, 1080, 1440, 1800, and 2400 minutes post infusion. In animals in which carotid and umbilical venous catheters were functional, samples were collected at -5, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330, and 360 minutes, but were not collected post-infusion. Amniotic and tracheal fluids were sampled at -5, 60, 120, 180, 240, 300, and 360 minutes during the infusion and at 60, 120, 180, 240, 360, 480, 720, 1080, 1440, 1800, and 2400 minutes post infusion. Cumulative collections of maternal urine were made at -5, 60, 120, 180, 240, 300, and 360 minutes during the infusion and at 60, 120, 180, 240, 360, 480, 720, 1080, 1440, 1800, and 2400 minutes post infusion; urine volume and pH were measured throughout the experimental period. Fetal urine was collected at -5, 60, 120, 180, 240, 300, and 360 minutes during the infusion and at 60, 120, 180, 240, 360, 480, and 720 minutes post infusion in a sterile 60 mL syringe from which the volume was measured. The fetal urine, except a 5 mL aliquot, was returned back to the amniotic cavity via the urine collection apparatus. At the end of each
sample, the fetal urine collection syringe was removed and replaced with a new sterile syringe. The concentrations of DPHM and $[^2\text{H}_{10}]$DPHM were measured in these samples using the GC-MS method described above (2.4.1.). The concentrations of DPMA and $[^2\text{H}_{10}]$DPMA were measured using the developed method described in section 2.4.2.

2.6.7. Sample Handling

Maternal and fetal blood samples were placed into heparinized glass tubes and gently mixed, with care taken to avoid contact of the blood with the rubber stopper of the Vacutainer®. These samples were then centrifuged at 3000 X g for 10 minutes. The plasma supernatant was removed and placed into clean borosilicate test tubes which were capped with PTFE-lined caps. Amniotic and tracheal fluid samples, and fetal and maternal urine samples were placed directly into clean borosilicate test tubes which were capped with PTFE-lined caps. All samples were stored frozen at -20° C until the time of analysis ($\leq$ 3 months from sample collection).

2.7. In Vitro Experiments

2.7.1. DPHM Blood Cell Uptake

Two experiments were conducted; one measured the time course necessary to attain equilibrium for blood cell (BC) uptake of DPHM, and the second experiment measured the effect of temperature on the uptake of DPHM into the BCs. The experimental protocol for the uptake studies involved spiking heparinized whole blood from non-pregnant ewes to obtain a total DPHM concentration of 2.0 μg/mL, incubating the samples at 39° C for designated periods of time, followed by immediate separation of plasma from the BCs by centrifugation. All samples
were stored frozen at -20° C prior to analysis. The concentration of DPHM was measured in both the BC and plasma fraction using a previously published GC-NPD method (Yoo et al., 1986). The ratio of DPHM concentration in plasma to BC was plotted as a function of time to determine the extent and rate of DPHM uptake into BC.

The experiment investigating the effect of temperature on DPHM uptake into BCs was to ensure that sample handling techniques (i.e., allowing the collected samples to cool to room temperature prior to separating the BC and plasma fractions) did not change the plasma DPHM concentration due to BC uptake. Whole blood was spiked to yield a total drug concentration of 2.0 µg/mL. One group of samples was incubated at 22° C while the second group of samples was incubated at 39° C for 30 minutes. Following incubation, the samples were immediately centrifuged at 3000 X g for 2 minutes, and the plasma and BC fractions were separated. Prior to analysis, these samples were stored frozen at -20° C. The concentration of DPHM in both the plasma and BC fraction in these samples was determined, using a previously published GC-NPD method (Yoo et al., 1986).

2.7.2. Adult and Fetal Hepatic Microsomal Metabolism Experiments

2.7.2.1. Preparation of Adult and Fetal Hepatic Microsomes

Adult hepatic microsomes were prepared from yearling male lambs (roughly 1 year old) at the time of slaughter at Pitt Meadows Meats, Pitt Meadows, B.C., Canada. The animals were stunned and then killed by exsanguination. The animals were disemboweled shortly after they were killed (2-5 minutes). The livers were removed and a lateral slice was made across the liver. The liver slice was immediately rinsed and placed into ice cold Tris KCl buffer pH 7.4 for transportation. Fetal hepatic microsomes were prepared from fetal lambs at the time of cesarean
section. The ewes were anesthetized as described in section 2.4.3.2. The sex, gestational age, and fetal weight are shown in Appendix 1. A midline incision was made, and the uterus exposed. An incision was made into the uterus and the fetus was exposed. A midline incision was made on the fetus and the fetal liver exposed. The umbilical vein was clamped and 2.5 mL of Euthanol® was immediately injected into the fetal heart. Following fetal death, the liver was immediately removed, cut into slices, rinsed free of blood, and immersed in ice cold Tris KCl pH 7.4 buffer for transport. Following the cesarean section, the uterine incision was closed and the abdomen of the ewe closed as described in section 2.4.3.2. The livers were again rinsed with ice cold Tris KCl pH 7.4 buffer to remove any remaining traces of blood, and then minced. All procedures were conducted on ice. The minced liver tissue (fetal or adult) was then homogenized in Tris KCl pH 7.4 buffer with a Potter-Elvehjem tissue grinder. A total of ten homogenization passes were made; five with the loose-fitting pestle for the initial homogenization, and five passes with the tight fitting pestle for the final homogenization. The homogenate was transferred to low speed centrifuge tubes and centrifuged at 9000 X g for 20 minutes at 4° C. The supernatant was filtered through four layers of “cheese” cloth. The supernatant was then transferred to high speed centrifuge tubes and the pellet from the low speed spin was discarded. The supernatant was centrifuged at 105,000 X g for 60 minutes at 4° C. The resulting supernatant was discarded and the lipid was wiped from the tube. The pellet was carefully resuspended to avoid the inclusion of the glycogen portion of the pellet and placed in a clean homogenization tube. The microsomal pellet was resuspended in EDTA/KCl pH 7.4 buffer with five passes of the loose fitting pestle. The resulting homogenate was transferred to a high speed centrifuge tube and centrifuged at 105,000 X g for 60 minutes at 4° C. The supernatant was discarded and the lipid wiped from the inside of the centrifuge tube. The pellet
was resuspended in (four equivalent volumes to pellet) 0.25 M sucrose solution and homogenized with four passes with the loose fitting pestle. The homogenate was transferred into cryo-test tubes and stored at -70°C until use.

2.7.2.2. Protein Concentration and Cytochrome P450 Measurements

The protein concentration of the adult and fetal microsomal preparations was determined using a modified Lowry method (Markwell et al., 1978). Cytochrome P450 was determined using a spectrophotometric assay based on the absorption of Cytochrome P450 bound to carbon monoxide, as described by Omaru et al., 1968.

2.7.2.3. DPHM and N-demethyl DPHM Quantitation

The quantitation of DPHM and N-demethyl DPHM was carried out using methodology described earlier (Abernethy and Greenblatt, 1983, and Blyden et al., 1986), with some modification. GC-MS analytical instrumentation was used rather than GC-NPD. Re-optimization of this methodology resulted in similar extraction and instrumentation parameters as those used in the original GC-MS assay for [²H₁₀]DPHM and DPHM. Fragment ions m/z 165 and 167 were used to quantitate DPHM and N-demethyl DPHM. The standard curve for N-demethyl DPHM used concentrations of 2.5 -50.0 ng/mL.
2.7.2.4. Fetal and Adult Hepatic Microsomal Incubations

Initial experiments to optimize conditions for microsomal incubations were carried out in the adult hepatic microsomes. These experiments included optimization of the amount of microsomal protein to be added, and optimization of the amount of substrate to be added to the incubation mixture. The microsomal incubations were conducted in a clean test-tube using 0.5 mL of 0.2 M phosphate pH 7.4 buffer, 10 μL of 300 mM MgCl₂, 1 mg of microsomal protein, and 10 μL of both 10 mM NADPH and NADH. The reaction mixture was then made up to volume (so that the final volume would be 1.0 mL) with distilled water. The test-tube containing the microsomal suspension was gently mixed, to avoid frothing, on a vortex mixer, and placed in a water bath at 39°C for 15 minutes as a pre-incubation. The reaction was started with the addition of 0.5 μmoles of substrate. The reaction was terminated at 0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 30.0, 45.0, and 60.0 minutes by the addition of 0.5 mL 1% ice cold HCl. The tubes were then immediately capped and frozen until the time of analysis. These tubes were assayed for DPHM and N-demethyl DPHM. Additional incubations were conducted using normal and boiled (control) adult and fetal microsomes. These incubations were conducted as outlined above, but were allowed to continue for 90 minutes. They were then assayed for both N-demethyl DPHM and DPMA.

2.7.3. Plasma Protein Binding of DPMA

The plasma protein binding of DPMA was measured in fetal and maternal plasma using equilibrium dialysis [Plexi-glass® dialysis cells (1.0 mL capacity) with cellophane dialysis membranes (molecular cut-off 12,000 daltons, Sigma Chemical Co., St. Louis, MO, U.S.A.)].
Maternal and fetal plasma for these experiments were obtained from two additional sheep set up for other experiments. The fetal and maternal drug-free plasma was obtained on non-experiment days and pooled. Aliquots of fetal and maternal plasma were spiked with DPHM and DPMA to yield total drug and metabolite concentrations of 1.0 μg/mL. Spiked fetal and maternal plasma was dialyzed against 0.1 M phosphate pH 7.4 buffer. Dialysis was carried out in a temperature controlled water bath at 39°C. Time-to-equilibrium studies were conducted with sampling times of 2, 4, 6, 8, 12, 24, and 36 hours. In each sample, the pH and the post dialysis volume were measured. Dialyzed buffer and plasma were transferred to clean borosilicate test-tubes, capped with PTFE-lined lids, and frozen at -20°C. Non-specific binding was determined by dialyzing blank buffer against spiked buffer (1.0 μg/mL). Concentrations of DPMA were measured in both buffer samples; the sum of these samples was then compared to the spiked metabolite concentration. The difference was taken as the extent of non-specific binding. The plasma protein binding of DPMA in pooled fetal and maternal plasma was determined in five replicates by dialyzing spiked fetal and maternal plasma against blank buffer for 8 hours. Then both buffer and plasma were removed, volumes and pH measured, and the samples stored as described above. DPMA concentrations were determined in both plasma and buffer samples. The free fraction was taken as the ratio of free metabolite concentration (buffer) over total metabolite concentration (plasma).
2.8. **Data Analysis**

2.8.1. **Data Reduction**

Data obtained from fetal monitoring was reduced in the following fashion. The data was obtained as minute averages on disk. This data was transferred to a spread-sheet (Microsoft Excel®), and 10 minute averages were calculated; the results are presented as the mean ± SEM, unless otherwise stated.

2.8.2. **Calculation of *In Utero* Fetal Weight**

Fetal weights *in utero* at the time of experimentation were estimated from the weight at birth and the time interval between the experiment and the birth using equation 1 (Gresham *et al.*, 1972):

\[
\text{Eq 1: } \log(\text{fetal weight } \textit{in utero}) = \log(\text{birth weight}) - 0.153 \times (\text{number of days between experiment and birth}).
\]

2.8.3. **Pharmacokinetic Data Analysis**

The model used to fit the data obtained from the 1.0 minute infusion was a 2 compartment open model with infusion input and elimination occurring from the central compartment shown below (Figure 5) (Gibaldi and Perrier, 1982).
Figure 5: A 2 compartment open model for the disposition of DPHM in fetal or maternal sheep.

The equation for the plasma concentration of DPHM of $[^2H_{10}]$DPHM is shown in equation 2:

Eq 2: $C_p = \frac{K_o(K_{21}-\alpha)(1-e^{-\alpha T})e^{-\alpha t} + K_o(\beta-K_{21})(1-e^{-\beta T})e^{-\beta t}}{V_{c_{app}}(\alpha-\beta)} + \frac{V_{c_{app}}(\alpha-\beta)}{V_{c_{app}}(\alpha-\beta)}$

where $T$ is the duration of the infusion, and is equal to $t$ during the duration of the infusion, and following the infusion $T$ is a constant equal to the total duration of the infusion (1.0 minute).

This equation gives $C_p$ (the plasma concentration at a particular time ($t$)), where $\alpha$ and $\beta$ are disposition constants, $V_{c_{app}}$ is the apparent volume of distribution of the central compartment, and $K_{12}$ and $K_{21}$ are the rate constants describing the transfer of drug from the central to peripheral and peripheral to the central compartments, respectively. $K_o$ is the infusion rate. The concentration vs. time data was fit using the ADAPT II computer fitting program. A maximum likelihood fitting algorithm with a power variance model was utilized, as shown in equation 3,
where δ and γ are constants describing the variance of the measured concentration (Cp) (D'Argenio and Schumitzky, 1988).

\[
\text{Eq. 3: } \text{Var} = \delta^2 \text{Cp}^\gamma
\]

Cp max was extrapolated from the fitted equation to the end of the infusion (i.e., 1.0 minute.). The area under the plasma concentration vs. time curve (AUC\(_0\rightarrow\infty\)) was calculated using a hybrid trapezoidal method. The linear trapezoidal method was used to calculate the AUC from time zero to the maximum plasma concentration one minute following the infusion (AUC\(_0\rightarrow\text{Cp max}\)), followed by the log-linear trapezoidal method to calculate the AUC from maximum plasma concentration to the last measured plasma concentration (AUC\(_\text{Cp max} \rightarrow \text{Cp last}\)). Finally, the AUC under the terminal portion of the disposition curve (AUC\(_{\text{Cp last} \rightarrow \infty}\)) was calculated as Cp last/β. The total AUC\(_0\rightarrow\infty\) was calculated as shown by equation 4 (Purves, 1992):

\[
\text{Eq 4: } AUC_{0\rightarrow\infty} = AUC_{0\rightarrow\text{Cp max}} + AUC_{\text{Cp max} \rightarrow \text{Cp last}} + AUC_{\text{Cplast} \rightarrow \infty}
\]

The area under the first moment curve (AUMC) was calculated in a similar fashion, that is, a hybrid method of linear and log-linear trapezoidal functions was used. The total body clearance (CL\(_T\)) was calculated as shown in equation 5 (Gibaldi and Perrier, 1982):

\[
\text{Eq. 5: } CL_T = \text{Dose}/AUC_{0\rightarrow\infty}
\]
The steady state volume of distribution (Vd_{ss}) was calculated as shown in equation 6 (Perrier and Mayersohn, 1982):

Eq. 6: \[ Vd_{ss} = \frac{\text{Dose}}{\text{AUC}_{0\rightarrow\infty}} \times \text{MDRT} \]

The volume of distribution area (Vd_{β}) was calculated as shown in equation 7 (Gibaldi and Perrier, 1982):

Eq. 7: \[ Vd_{\beta} = \frac{\text{Dose} \times \beta}{\text{AUC}_{t\rightarrow\infty}} \]

The total mean residence time (MTRT) was calculated as shown in equation 8 (Weiss, 1992):

Eq. 8: \[ \text{MTRT} = \frac{\text{AUMC}_{0\rightarrow\infty}}{\text{AUC}_{0\rightarrow\infty}} \]

The mean disposition residence time (MDRT) is calculated as shown in equation 9 (Weiss, 1992), where T/2 is the correction factor for drug administration by infusion:

Eq. 9: \[ \text{MDRT} = \text{MTRT} - \frac{T}{2} \]

The systemic availability (F) of DPHM or [^{2}H_{10}]DPHM administered either via the mesenteric vein in adult sheep or via the umbilical vein in the fetal lambs (i.e., the test routes) was calculated as shown in equation 10 (Gibaldi and Perrier, 1982):
Eq. 10: \[ F = \frac{\text{AUC}_{0-\infty} \text{(test route)}}{\text{AUC}_{0-\infty} \text{(control route)}} \]

The fetal total body clearance (CL\text{f}) following simultaneous infusion to steady-state was calculated as shown in equation 11 (Gibaldi and Perrier, 1982):

Eq. 11: \[ \text{CL}_f = \frac{K_o}{C_{pss}} \]

Where C\text{pss} is the steady-state plasma concentration.

The fetal or maternal plasma concentration vs. time for the simultaneous maternal and fetal infusion experiments was fit using a 2 compartment open model with elimination occurring from the central compartment (Gibaldi and Perrier, 1982) (Figure 5). The concentration vs. time data was fit using the ADAPT II computer fitting program using a maximum likelihood fitting algorithm (variance model for Cp; Var = \delta*(C_p) + \gamma, where \delta and \gamma are estimated variance coefficients) (D’Argenio and Schumitzky, 1988). C\text{p}_{0} was extrapolated from the fitted equation. The area under the plasma concentration vs. time curve (AUC\text{0-\infty}) was calculated using the linear trapezoidal method (Gibaldi and Perrier, 1982).

A 2 compartment-open model was used to describe the disposition of DPHM and [2H\text{10}]DPHM in the maternal and fetal sheep, respectively (Figure 6). This model assumes steady-state plasma concentrations, and that drug elimination occurs from both the maternal and fetal compartments. Trans- and non-placental clearances of DPHM were calculated as described (Szeto, 1982, and Szeto et al., 1982) in equations 13-18 below:
Figure 6: Schematic representation of the 2 compartment open model for drug disposition in the maternal fetal unit.

Eq. 13: \[ CL_{mm} = \frac{K_o}{[C_{mss} - C_{fss}*(C_{mss}'/C_{fss}')] } \]

Eq. 14: \[ CL_{ff} = \frac{K_o'}{[C_{fss}' - C_{mss}^* *(C_{fss}/C_{mss})] } \]

Eq. 15: \[ CL_{mf} = CL_{ff} *(C_{fss}/C_{mss}) \]

Eq 16: \[ CL_{fm} = CL_{mm} *(C_{mss}'/C_{fss}') \]

Eq 17: \[ CL_{mo} = CL_{mm} - CL_{mf} \]

Eq 18: \[ CL_{fo} = CL_{ff} - CL_{fm} \]
where CLmm and CLff are the total body clearances from the mother and fetus, respectively, CLmf is the trans-placental clearance from the maternal to fetal compartment, CLfm is the trans-placental clearance from the fetal to the maternal compartment, and CLmo and CLfo are the non-placental clearances from the mother and fetus, respectively. By using the values for the infusion rates to the mother (Ko) and fetus (Ko') and the steady-state drug concentrations in the mother and fetus following maternal infusion (Cmss and Cfss) and fetal infusion (Cmss' and Cfss'), all the clearances were calculated. Trans-placental clearances were also calculated using the Fick method in two animals in which umbilical flow and umbilical venous concentrations of DPHM and [²H₁₀]DPHM were measured. The calculations for the trans-placental clearances are shown in equations 19 and 20 below:

Eq. 19: $CL_{mf} = Q_{um} \times (C(DPHM)_{uv} - C(DPHM)_{fa})/C(DPHM)_{fa}$

Eq. 20: $CL_{fm} = Q_{um} \times (C([²H₁₀]DPHM)_{fa} - C([²H₁₀]DPHM)_{uv})/C([²H₁₀]DPHM)_{fa}$

In addition, this 2 compartment model was also used with the integrated form, using the mass balance approach (Edling and Jusko, 1986). The various trans- and non-placental clearances were calculated as shown in equations 21-24 below:

Eq. 21: $CL_{mo} = Dose(M) \times AUC_{ff} - Dose(F) \times AUC_{mf} - \frac{AUC_{mm} \times AUC_{ff} - AUC_{mf} \times AUC_{fm}}{AUC_{mm} \times AUC_{ff} - AUC_{mf} \times AUC_{fm}}$
Eq. 22: \[ CL_{fo} = \frac{Dose(F) \cdot AUC_{mm} - Dose(M) \cdot AUC_{fm}}{AUC_{mm} \cdot AUC_{ff} - AUC_{mf} \cdot AUC_{fm}} \]

Eq. 23: \[ CL_{mf} = \frac{Dose(F) \cdot AUC_{mf}}{AUC_{mm} \cdot AUC_{ff} - AUC_{mf} \cdot AUC_{fm}} \]

Eq. 24: \[ CL_{fm} = \frac{Dose(M) \cdot AUC_{fm}}{AUC_{mm} \cdot AUC_{ff} - AUC_{mf} \cdot AUC_{fm}} \]

where \( CL_{mo} \) and \( CL_{fo} \) are the elimination clearances of DPHM from the mother and fetus, respectively. \( CL_{mf} \) and \( CL_{fm} \) are the transfer clearances of DPHM from mother to fetus and fetus to mother, respectively. \( AUC_{ff} \) and \( AUC_{mm} \) are the areas under the plasma concentration vs. time curve of fetal DPHM following fetal administration and of maternal DPHM following maternal administration, while \( AUC_{mf} \) and \( AUC_{fm} \) are the areas of fetal DPHM following maternal administration and maternal DPHM following fetal administration, respectively. For the infusion data the total mean residence time (MTRT) was calculated as shown above; however, because this dosage form was administered both as a bolus and a simultaneous infusion, the mean disposition residence time (MDRT) is calculated as shown in equation 25:

Eq. 25: \[ MDRT = MTRT - \frac{\Sigma(\int_0^\infty X \, dt)}{\Sigma(dose)} \]

Where the correction factor \( \frac{\Sigma(\int_0^\infty X \, dt)}{\Sigma(dose)} \) for a loading dose bolus and simultaneous infusion is equal to \( (Ko \cdot T^2/2) \cdot (Ko \cdot T + Xo) \). The steady state volume of distribution \( (V_{dss}) \) was
calculated as shown in equation 7 (Perrier and Mayersohn, 1982). An additional correction was made to the fetal steady-state volume of distribution. This was to account for the portion of the fetal dose which is transferred from the fetus to the mother across the placenta. The fetal dose lost due to placental transfer (Dose') is calculated as shown in equation 26:

Eq. 26: \[ \text{Dose'} = CL_{mm} \times AUC_{fm} \]

The fraction of the fetal dose (f') which remains in the fetus is calculated as shown in equation 27:

Eq. 27: \[ f' = (\text{Total Fetal Dose} - \text{Dose'})/\text{Total Fetal Dose} \]

Thus, the corrected fetal apparent steady-state volume of distribution (Vd_{ss}') is calculated as shown in equation 28:

Eq. 28: \[ Vd_{ss}' = (f' \times \text{Total Fetal Dose}/AUC_{ff}) \times MDRT \]
Fetal and maternal plasma metabolite data was fit using the model outlined below (Figure 7). A linked pharmacokinetic model (i.e., where parent drug and metabolite data were simultaneously fit) was used to reduce the bias in the pharmacokinetic parameter estimates due to individually modeling parent drug and metabolite. A 1 compartment model was assumed from the metabolite, since the data obtained could not support a higher order model for the metabolite.

![Diagram of pharmacokinetic model](image)

**Figure 7:** A linked pharmacokinetic model showing 2 compartment kinetics for parent drug (DPHM), and 1 compartment kinetics for the metabolite (DPMA) used for the estimation of metabolite pharmacokinetic parameters in mother and fetus.

The plasma concentration data for DPHM and the corresponding metabolite was fit using the maximum likelihood algorithm with the ADAPT II computer fitting program to the differential equations 29-31 shown below (D'Argenio and Schumitzky, 1988).

**Eq. 29:** \[
\frac{dX(1)}{dt} = K_0 + K_{21}X(2) - (K_{12} + K_{10})X(1)
\]
Eq. 30: \[ \frac{dX(2)}{dt} = K_{12}X(1) - K_{21}X(2) \]

Eq. 31: \[ \frac{dX(3)}{dt} = F_mK_{10}X(1) - K_mX(3) \]

Where \( K_0 \) is the infusion rate of DPHM to mother and fetus, \( K_{21}, K_{12}, \) and \( K_{10} \) are the rate constants for the transfer of drug from the tissues to the central compartment, from the central compartment to the tissues, and from the central compartment out, respectively. \( K_m \) is the rate constant for the elimination of the metabolite DPMA. \( F_m = \frac{f_mV_m}{K_{10}}, \) where \( K_{10} = K_{out} + K_f \), and where \( V_m \) is the volume of distribution of the metabolite, and \( K_f \) is the formation rate constant for the metabolite. The output equations for the concentration of DPHM and DPMA are \( C_p(DPHM) = \frac{X(1)}{V_{c_{app}}} \) and \( C_p(DPMA) = X(3) \). The variance model for \( C_p(DPHM) \) and \( C_p(DPMA) \) used in the fitting of these equations were linear variance models (i.e., \( \text{Var}(DPHM) = C_p(DPHM)\delta(1) + \gamma(1) \), and \( \text{Var}(DPMA) = C_p(DPMA)\delta(2) + \gamma(2) \)).

The renal clearance of DPHM and DPMA in both mother and fetus were calculated as shown below in equations 32 and 33 (Gibaldi and Perrier, 1982):

Eq. 29: \[ \text{CL(DPHM)}_{\text{ren}} = \sum X_u(DPHM)/\text{AUC}_{0\rightarrow\infty}(DPHM) \]

Eq. 30: \[ \text{CL(DPMA)}_{\text{ren}} = \sum M_u(DPMA)/\text{AUC}_{0\rightarrow\infty}(DPMA) \]

where \( \sum X_u \) and \( \sum M_u \) are the total cumulative amount of DPHM or DPMA excreted in the urine, respectively.
2.8.4. **Statistical Analysis**

Values are expressed as mean values ± standard error of the mean (SEM). A paired sample T-test was conducted to test for differences in the ratio of the AUC\textsubscript{0→∞} of DPHM and \[^{2}\text{H}_{10}]\text{DPHM}, pharmacokinetic parameters for isotope effect control studies, and the pharmacokinetic parameters following the simultaneous maternal/fetal infusions (Zar, 1984). Where the requirements of a two sample T-test could not be assumed, the non-parametric equivalent (*i.e.*, Mann Whitney U-test) was used. The time required to reach steady-state was determined by using three groups of mean concentration values (*i.e.*, 150 and 180, 240 and 270, and 330 and 360 minutes) with a repeated measures ANOVA (Zar, 1984). Statistical differences between the fetal effects from the control periods and the test period were determined by repeated measures ANOVA (Zar, 1984).
3. Results

3.1. Development of Analytical Methodology

3.1.1. Capillary Gas Chromatographic-Mass Spectrometric Analysis of DPHM and $[^2\text{H}_{10}]$DPHM.

3.1.1.1. Optimization of Mass Spectrometer and Gas Chromatograph

DPHM and $[^2\text{H}_{10}]$DPHM both undergo extensive fragmentation following GC-MS/EI (Figure 8). These molecules appear to fragment at the ether linkage; hence, the molecular ions of either DPHM or $[^2\text{H}_{10}]$DPHM were absent. The fragmentation of DPHM and $[^2\text{H}_{10}]$DPHM results in three predominant mass to charge ratio ($m/\text{z}$) fragment ions; 58, 165, and 167 for DPHM, and 58, 173, and 177 for $[^2\text{H}_{10}]$DPHM. Selected ion monitoring (SIM) was used to optimize both the selectivity and sensitivity of this analytical method. Two fragment ion pairs (i.e., $m/\text{z}$ 167-DPHM and 177-$[^2\text{H}_{10}]$DPHM, and $m/\text{z}$ 165-DPHM and 173-$[^2\text{H}_{10}]$DPHM) allowed for differentiation between the labeled and unlabeled drugs, and showed the abundance necessary to achieve the required sensitivity. Ions $m/\text{z}$ 165 and 173 were ultimately monitored for the quantitation of DPHM and $[^2\text{H}_{10}]$DPHM. To further optimize the mass spectrometer, a manual tuning algorithm was employed. This tuning algorithm for the mass spectrometer uses the fragment ions $m/\text{z}$ 100, 131, and 219, which are derived from the tuning reagent PFTBA. This procedure further enhanced the sensitivity of the assay by 20 fold over the auto tune algorithm. The dwell time was set at 50 msec to provide at least 15 scans/chromatographic peak.
Figure 8: The mass spectra and fragment assignments of a) DPHM and b) $[^2\text{H}_{10}]$DPHM following electron impact ionization (70 eV) of the purified standards.
The fragmentation of N-demethyl DPHM resulted in a mass spectrum which was similar to that of DPHM, that is, predominant ions with a \( m/z \) of 165 and 167 were observed (Figure 9). In addition, this metabolite was found to co-elute with DPHM on a cross linked 5% phenylmethyl silicone coated capillary column, resulting in the inability to reliably quantitate the parent drug in the presence of the metabolite. To remedy this problem, a capillary column with a liquid phase coating of 7% cyanopropyl:5% phenylmethylsilicone (DB-1701) was chosen. On this column, the N-demethyl metabolites eluted between the internal standard and DPHM/\( [^{12}\text{H}_{10}] \)DPHM. Optimal column performance for the chromatography of DPHM and \( [^{12}\text{H}_{10}] \)DPHM was found to occur at a helium carrier gas flow corresponding to a column head pressure of 15 P.S.I. To enhance sensitivity, the splitless mode of sample introduction was chosen. Purge time (\( i.e., \) the time during which the volatilized sample is introduced onto the capillary column) of less than 1.25 minutes resulted in a decrease in sensitivity, while a purge time greater that 1.5 minutes did not offer a substantial increase in sensitivity (Figure 10). An optimal purge time of 1.5 minutes was chosen. Inlet temperatures between 200 and 275\( ^\circ \)C did not appear to affect the chromatography, or the apparent sensitivity of the assay method; therefore, an inlet temperature of 225\( ^\circ \)C was used. In contrast to inlet temperature, initial column temperature did have a substantial effect on the chromatography of DPHM and \( [^{12}\text{H}_{10}] \)DPHM (Figure 11). Temperatures below 140\( ^\circ \)C were found to result in wide peaks, complex (\( i.e., \) dirty) chromatograms, and decreased resolution between DPHM, \( [^{12}\text{H}_{10}] \)DPHM, the N-demethylated metabolites, and orphenadrine (the internal standard). An optimized initial column temperature of 140\( ^\circ \)C was used for the remainder of the assay development.
Figure 9: The mass spectrum and mass assignments of N-demethyl DPHM following GC-MS with electron impact ionization (70 eV).
The optimized chromatographic parameters and temperature program used for the assay resulted in retention times for DPHM and $[^2\text{H}_{10}]$DPHM of 7.67 and 7.64 minutes, respectively. The internal standard (orphenadrine) eluted at 8.05 minutes. This temperature program resulted in a total analysis time of 12.7 minutes.

3.1.1.2. Optimization of the Extraction Procedure for the DPHM and $[^2\text{H}_{10}]$DPHM Analysis Method.

The choice of the optimum solvent system for the extraction for the DPHM and $[^2\text{H}_{10}]$DPHM from biological samples was made on the basis of relative extraction efficiency, selectivity (lack of interfering chromatographic peaks), and ease of extraction. Both methylene chloride and 98% hexane: 2% isopropyl alcohol showed greater relative extraction efficiencies than either toluene or hexane (Figure 12). The 2% isopropyl alcohol: 98% hexane mixture appeared to be more selective than methylene chloride, since the chromatographic baselines of
blank biological matrices extracted with the former solvent were free from interfering peaks. In addition, the extraction conducted with the isopropyl alcohol:hexane mixture was easier, since the organic layer requiring transfer was on top of the aqueous layer, unlike with methylene chloride. The addition of 0.05 M TEA to the extraction solvent resulted in a large increase in the relative extraction efficiency (i.e., ~ a 4 fold increase) (Figure 12). Further increases in either isopropyl alcohol or TEA concentration in the extraction solvent did not result in an additional improvement in efficiency, nor did the use of silanized glassware.

Figure 11: The effect of varying the initial column temperature on the half-height peak width of DPHM.
The effect of mixing time on the relative extraction efficiency of DPHM with 0.05 M TEA in 2% isopropyl alcohol: 98% hexane was examined. The peak area counts of DPHM increased from 5 minutes to 15 minutes, but did not increase further following 15 minutes of extraction. An optimal mixing time of 20 minutes was chosen for the extraction of DPHM and $[^2\text{H}_{10}]$DPHM.

The optimized GC-MS parameters, in addition to the optimized extraction procedure (Figure 13), resulted in an analytical method which was free from chromatographic interference resulting from co-extracted endogenous materials (Figure 14). In addition, this method provided the necessary sensitivity and selectivity to quantitate both DPHM and $[^2\text{H}_{10}]$DPHM in biological samples obtained from pregnant sheep (i.e., amniotic fluid, fetal tracheal fluid, and plasma).
**BIOLOGICAL SAMPLE**
- Fetal or maternal plasma
- Amniotic fluid
- Fetal Tracheal fluid

Make up to 1.0 mL with distilled water
+ Internal Standard (orphenadrine 200 ng)
+ 0.5 mL 1N NaOH
+ 7.0 mL organic solvent
(0.05 M triethylamine in 2% isopropyl alcohol:98% hexane)

Mix 20 minutes

Freeze 10 minutes at -20°C

Centrifuge 10 minutes at 3000 x g

Aqueous (Waste)  Organic

Evaporate to dryness under N2 at 30°C.

Reconstitute with 0.15 mL
0.05M Triethylamine in toluene

2.0 μL for injection.

Figure 13: Optimized extraction procedure for DPHM and [²H₁₀]DPHM from biological fluids obtained from the pregnant sheep.
Figure 14: Ion chromatograms for DPHM (m/z 165) and [2H10]DPHM (m/z 173) in plasma, fetal tracheal fluid, and amniotic fluid. Blanks, spiked standards (2.0 ng/mL), and biological samples are shown.
3.1.1.3. Calibration Curve for the DPHM and $[^2\text{H}_{10}]$DPHM Assay.

The calibration curves for DPHM and $[^2\text{H}_{10}]$DPHM showed good linearity over the range from 2.0 ng/mL to 200.0 ng/mL in all of the biological matrices examined. A sample calibration curve from plasma is shown in figure 15. The coefficients of variation did not exceed 10% (C.V.) for each point of the calibration curve in plasma, fetal tracheal fluid, and amniotic fluid. The regression coefficients in plasma, fetal tracheal fluid, and amniotic fluid were, in most instances, greater than 0.999. The weighting function $1/Y^2$ was used. The regression equation for DPHM was $Y = 0.0049X + (-0.0016)$, and for $[^2\text{H}_{10}]$DPHM was $Y = 0.0033X + (-0.0013)$. The slope of the DPHM calibration curve was greater than the slope for the $[^2\text{H}_{10}]$DPHM calibration curve, reflecting the difference in the relative abundance of the fragment ions $m/z$ 165 and 173, respectively (Figure 8).

![Calibration curve for DPHM and $[^2\text{H}_{10}]$DPHM in plasma (Mean ± S.D.)](image-url)
3.1.1.4. Extraction Recoveries of DPHM and [\(^2\)H\(_{10}\)]DPHM.

The extraction recovery of DPHM and [\(^2\)H\(_{10}\)]DPHM following liquid-liquid extraction from ovine plasma, fetal tracheal fluid, and amniotic fluid using 0.05 M TEA in 98% hexane: 2% isopropyl alcohol was nearly complete. No apparent concentration dependent changes in the extraction recoveries were noted, since the extraction recovery at the three different concentrations tested (i.e. 2.0, 50.0, and 200.0 ng/mL) were the same. The mean (± S.D.) recovery of DPHM and [\(^2\)H\(_{10}\)]DPHM from plasma was 98 ± 2 and 105 ± 3%, from amniotic fluid was 100 ± 5 and 110 ± 6%, and from fetal tracheal fluid was 97 ± 5 and 104 ± 5%, respectively.

3.1.1.5. Sample Stability Assessment of DPHM and [\(^2\)H\(_{10}\)]DPHM.

The samples containing DPHM and [\(^2\)H\(_{10}\)]DPHM stored in the freezer at -20° C appeared stable for up to 12 months, since the concentrations of drug measured in these samples did not deviate from the known concentration. Following three freeze-thaw cycles, no differences in the concentrations of DPHM and [\(^2\)H\(_{10}\)]DPHM were noted between the control and freeze-thaw samples (Mann-Whitney U test, P>0.05) [See Figure 16]. The slope of the linear regression of the measured concentration of plasma DPHM and [\(^2\)H\(_{10}\)]DPHM vs. time was not significantly different from zero (Two Sample T-test; P> 0.05) following bench-top storage for up to and including 24 hours (See Figure 17). The final sample stability test was conducted with the extracted samples on the auto-sampler tray of the GC-MS.
Figure 16: The effect of three freeze-thaw cycles on the plasma concentrations of DPHM and $[^2\text{H}_{10}]$DPHM in spiked plasma samples (Mean ± S.D., n=4).

Figure 17: The effect of prolonged bench-top storage (22° C) on the plasma concentrations of DPHM and $[^2\text{H}_{10}]$DPHM in spiked plasma samples (n=2).
The extracted samples were periodically injected into the GC-MS during large sequence runs for a period of up to 72 hours. No differences in the ratios of DPHM and $[^2\text{H}_{10}]$DPHM were detected.

### 3.1.1.6 Validation of DPHM and $[^2\text{H}_{10}]$DPHM Gas Chromatographic - Mass Spectrometric Analysis Method.

The validation of this method involved estimation of both intra- and inter-day variability. In addition, the assay was cross validated with a previously published method for the quantitation of DPHM (Yoo et al., 1986). The estimates of intra-day variability for DPHM and $[^2\text{H}_{10}]$DPHM were below 17% at 2.0 ng/mL, and below 8% at all other concentrations examined in all three biological matrices tested (Table 1). The measured inter-day variability for DPHM and $[^2\text{H}_{10}]$DPHM was below 15% at 2.0 ng/mL and below 10% for all other points (Table 2). A published GC-NPD method was used in the cross validation studies (Yoo et al., 1986). The concentrations of DPHM and $[^2\text{H}_{10}]$DPHM were determined independently, since the GC-NPD could not differentiate between DPHM and $[^2\text{H}_{10}]$DPHM. When the concentrations of DPHM and $[^2\text{H}_{10}]$DPHM measured by the GC-NPD method were plotted against the concentrations of DPHM and $[^2\text{H}_{10}]$DPHM independently measured by the GC-MS method, the correlation was excellent ($r=1.000$ DPHM and $r=0.999$ $[^2\text{H}_{10}]$DPHM) (Figure 18).
Table 1: Intra-day variability of DPHM and $[^{2}H_{10}]$DPHM measurements in plasma, amniotic, and fetal tracheal fluid.

<table>
<thead>
<tr>
<th>DPHM</th>
<th>Plasma</th>
<th>Amniotic</th>
<th>Tracheal</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 ng/mL</td>
<td>2.1 ± 0.2</td>
<td>1.9 ± 0.32</td>
<td>1.9 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>(9.1%)</td>
<td>(16.5%)</td>
<td>(10.4%)</td>
</tr>
<tr>
<td>20.0 ng/mL</td>
<td>18.7 ± 0.5</td>
<td>17.7 ± 1.3</td>
<td>18.1 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>(2.9%)</td>
<td>(7.4%)</td>
<td>(6.2%)</td>
</tr>
<tr>
<td>100.0 ng/mL</td>
<td>107.2 ± 2.8</td>
<td>100.6 ± 3.3</td>
<td>106.6 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>(2.6%)</td>
<td>(3.3%)</td>
<td>(3.4%)</td>
</tr>
<tr>
<td>200.0 ng/mL</td>
<td>214.8 ± 5.2</td>
<td>212.3 ± 10.4</td>
<td>208.8 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>(2.4%)</td>
<td>(4.9%)</td>
<td>(0.7%)</td>
</tr>
<tr>
<td>$[^{2}H_{10}]$DPHM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0 ng/mL</td>
<td>2.2 ± 0.3</td>
<td>1.9 ± 0.2</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>(13.8%)</td>
<td>(10.1%)</td>
<td>(16.4%)</td>
</tr>
<tr>
<td>20.0 ng/mL</td>
<td>19.3 ± 0.6</td>
<td>16.3 ± 0.9</td>
<td>17.9 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>(3.1%)</td>
<td>(5.4%)</td>
<td>(8.1%)</td>
</tr>
<tr>
<td>100.0 ng/mL</td>
<td>108.6 ± 3.7</td>
<td>100.2 ± 1.1</td>
<td>106.0 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>(3.4%)</td>
<td>(1.1%)</td>
<td>(3.2%)</td>
</tr>
<tr>
<td>200.0 ng/mL</td>
<td>214.6 ± 6.0</td>
<td>207.4 ± 9.7</td>
<td>200.4 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>(2.8%)</td>
<td>(4.7%)</td>
<td>(1.1%)</td>
</tr>
</tbody>
</table>

Table 2: Inter-day variability of DPHM and $[^{2}H_{10}]$DPHM measurements in plasma, amniotic, and fetal tracheal fluid.

<table>
<thead>
<tr>
<th>DPHM</th>
<th>Plasma</th>
<th>Amniotic</th>
<th>Tracheal</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0 ng/mL</td>
<td>2.2 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>(7.5%)</td>
<td>(6.0%)</td>
<td>(14.4%)</td>
</tr>
<tr>
<td>20.0 ng/mL</td>
<td>20.6 ± 1.6</td>
<td>16.0 ± 1.0</td>
<td>20.5 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>(7.9%)</td>
<td>(6.4%)</td>
<td>(7.4%)</td>
</tr>
<tr>
<td>100.0 ng/mL</td>
<td>119.4 ± 6.9</td>
<td>90.6 ± 3.2</td>
<td>113.6 ± 9.2</td>
</tr>
<tr>
<td></td>
<td>(5.8%)</td>
<td>(3.4%)</td>
<td>(8.1%)</td>
</tr>
<tr>
<td>200.0 ng/mL</td>
<td>233.2 ± 17.9</td>
<td>185.0 ± 3.5</td>
<td>229.8 ± 21.8</td>
</tr>
<tr>
<td></td>
<td>(7.7%)</td>
<td>(1.9%)</td>
<td>(9.5%)</td>
</tr>
<tr>
<td>$[^{2}H_{10}]$DPHM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0 ng/mL</td>
<td>2.4 ± 0.2</td>
<td>1.7 ± 0.1</td>
<td>2.1 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>(8.1%)</td>
<td>(7.2%)</td>
<td>(12.3%)</td>
</tr>
<tr>
<td>20.0 ng/mL</td>
<td>19.7 ± 1.5</td>
<td>15.8 ± 1.4</td>
<td>19.9 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>(7.4%)</td>
<td>(9.0%)</td>
<td>(5.7%)</td>
</tr>
<tr>
<td>100.0 ng/mL</td>
<td>108.1 ± 8.1</td>
<td>87.6 ± 3.9</td>
<td>91.6 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>(7.5%)</td>
<td>(4.5%)</td>
<td>(7.1%)</td>
</tr>
<tr>
<td>200.0 ng/mL</td>
<td>217.2 ± 14.8</td>
<td>179.6 ± 4.8</td>
<td>221.2 ± 17.3</td>
</tr>
<tr>
<td></td>
<td>(6.8%)</td>
<td>(2.7%)</td>
<td>(7.8%)</td>
</tr>
</tbody>
</table>

Mean Area Ratios ± SD, n=6
Numbers in brackets are the coefficients of variation (%)
The minimal detectable concentration of DPHM and $[^2\text{H}_{10}]$DPHM was 0.5 ng/mL (6.7 pg of the analyte at the detector). This corresponds to a signal to noise ratio (S/N) of $>3$. The minimal quantifiable concentration was 2.0 ng/mL (i.e., 27.6 pg of the analyte at the detector). The minimal quantifiable concentration also fell within the acceptable limits of inter- and intra-day variability (i.e., $<20\%$ relative standard deviation for the lowest concentration) (Shah et al., 1992).
3.1.2. Capillary Gas Chromatographic - Mass Spectrometric Analysis of DPMA and $[^2\text{H}_{10}]$DPMA

3.1.2.1. Optimization of Mass Spectrometer and Gas Chromatograph

The mass spectra of the tert-butyldimethylsilyl (TBDMS) derivatives of DPMA and $[^2\text{H}_{10}]$DPMA following GC-MS/EI showed extensive fragmentation, resulting in numerous small fragment ions with no molecular ion present at $m/z$ 356 for DPMA and at $m/z$ 366 for $[^2\text{H}_{10}]$DPMA (Figure 19). The prominent ions which also retained the stable isotope label for the TBDMS derivatives of DPMA and $[^2\text{H}_{10}]$DPMA were $m/z$ 167 and 183, and $m/z$ 177 and 193, respectively. Fragment ions of low intensity corresponding to [M-57]$^+$ or the loss of the tert-butyl group from the derivatized DPMA and $[^2\text{H}_{10}]$DPMA (i.e., $m/z$ 299 and 309) were also observed (Figure 19). The fragmentation of the tert-butyldimethylsilyl derivative of the internal standard, DPAA, resulted in a base fragment ion [M-57]$^+$ of $m/z$ 269, and a smaller fragment ion at $m/z$ 165. SIM was used to optimize both the sensitivity and selectivity of this assay. Initially fragment ions $m/z$ 183 and 193 were monitored for the quantitation of DPMA and $[^2\text{H}_{10}]$DPMA, respectively. However, the fragmentation of DPMA also yielded a small fragment at $m/z$ 193 which resulted in chromatographic interference when measuring $[^2\text{H}_{10}]$DPMA, particularly at lower concentrations. Next, the ion pair $m/z$ 167 and 177 was selected for SIM quantitation. The ion chromatogram at $m/z$ 177 resulted in a clean chromatogram at the retention time for $[^2\text{H}_{10}]$DPMA. On the other hand, the ion chromatogram for $m/z$ 167 resulted in considerable interference from co-extracted components in adult sheep urine, which could not be eliminated either through GC oven programming or sample clean-up using liquid-liquid extraction. Thus, the fragment ions $m/z$ 183 and 177 were monitored for DPMA and $[^2\text{H}_{10}]$DPMA, respectively. Although the fragment ion $m/z$ 269 was much more prominent than others in the mass spectrum of the internal standard, DPAA, there was considerable interference in the ion chromatogram. Thus, the fragment ion $m/z$ 165 was monitored for the quantitation of DPAA.
Figure 19: The mass spectra of the TBDMS derivatives of DPMA and \(^{[2}H_{10}]DPMA\) and the mass fragment assignments following GC-MS with electron impact ionization (70 eV) of the standards.
The optimization of the mass spectrometer tuning was achieved using the manual tuning option to select the fragment ions m/z 100, 131, and 219 of PFTBA. This further enhanced the sensitivity of detection of DPMA and [\(^2\text{H}_{10}\)]DPMA to the level required for the assay. The dwell time for SIM analysis was set a 125 milliseconds to provide at least 15 scans per chromatographic peak.

As with the previous analytical method, two columns were examined for optimum chromatographic resolution between the analytes (i.e., DPMA, [\(^2\text{H}_{10}\)]DPMA, and DPAA) and endogenous co-extracted components. The Ultra-2 column provided optimum peak shape and resolution of the analytes from endogenous components. The peak shapes of DPMA and [\(^2\text{H}_{10}\)]DPMA were improved, and the peak width reduced by increasing the helium column head pressure from 7.5 to 15.0 P.S.I. (i.e., increasing linear gas velocity). Increasing the initial column temperature from 150° C to 200° C resulted in shorter elution times for DPMA and [\(^2\text{H}_{10}\)]DPMA (i.e., 13.5 vs. 8.5 minutes); however, significant peak broadening and tailing was observed. An optimum initial column temperature of 150° C was thus chosen. Increasing the injector temperature did not result in significant increases in the peak areas, but helped to overcome a “carry-over” phenomenon, which was noted following the injection of MTBSTFA derivatized DPMA and [\(^2\text{H}_{10}\)]DPMA at lower injector temperatures. The injection of blank toluene would not result in carry-over; however, the injection of toluene treated with MTBSTFA resulted in a ghost-peak of DPMA and [\(^2\text{H}_{10}\)]DPMA. This problem was overcome by increasing the injector temperature from 225 to 280° C. These GC-MS conditions resulted in ion chromatograms free from interference from co-eluting peaks in urine and plasma. The ion chromatograms of plasma and of urine spiked with 250 ng/mL each of DPMA, [\(^2\text{H}_{10}\)]DPMA, and 400 ng/mL of DPAA and the corresponding blank matrices can be seen in figure 20. It should be noted that the Y-axis for the blank biological samples is amplified to better show the ion chromatograms of the extracted blank matrices.
Figure 20: Ion chromatograms of m/z 165 (internal standard), 183 (DPMA), and 177 ([2H10]DPMA) in blank plasma and urine, and plasma and urine spiked with 250.0 ng/mL of DPMA, 250 ng/mL of [2H10]DPMA, and 400 ng/mL of the internal standard DPAA.

I.S. - Internal Standard for analysis method

*Note: The y-axis scaling is increased in the blank to show more clearly the base-line of the ion chromatogram.
3.1.2.2. Optimization of Extraction and Derivatization Procedures for DPMA and \[^{2}H_{10}\]DPMA.

Ethyl acetate and toluene resulted in similar relative extraction efficiencies for DPMA and \[^{2}H_{10}\]DPMA, but the ion chromatograms were cleaner following toluene extraction compared to ethyl acetate. Although toluene resulted in greater emulsion formation following mixing compared to ethyl acetate, toluene was subsequently used as the optimized extraction solvent due to the ease of "cracking" the emulsion upon cooling. The mixing time was optimized to 20 minutes. DPMA and \[^{2}H_{10}\]DPMA were derivatized with both PFBBr or MTBSTFA to form PFB or TBDMS derivatives, respectively. The TBDMS derivatives provided better response using EI mass spectrometry, while the PFB derivatives provided better responses in NCI mass spectrometry. TBDMS derivatives were utilized because GC-MS/EI was to be used for analysis, and furthermore, the derivatization procedure was rapid and easier. No difference in peak area response was noted with the different derivatization incubation times (i.e., 30, 60, and 90 minutes) at 60° C, thus, a time of 60 minutes was chosen as optimal. In addition, with differing volumes of MTBSTFA (i.e., 10, 25, 50, and 100 μL), the peak areas were equivalent, suggesting that the volume of derivatizing agent did not influence the sensitivity of the method. The volume ultimately chosen for the remainder of the assay development was 25 μL. The optimized extraction and derivatization procedure for DPMA and \[^{2}H_{10}\]DPMA is shown in figure 21.
Biological Sample
- fetal or maternal plasma
- fetal or maternal urine

Make up to 1.0 mL with distilled water

Add internal standard (400 ng) DPAA
Add 0.4 mL 1.0 M HCl
Add 5.0 mL Toluene

Mix for 20 minutes

Freeze for 10 minutes at -20°C

Centrifuge for 10 minutes at 3000 X g

Discard Aqueous layer
Transfer organic layer to clean test tube

Evaporate to dryness under a gentle N2 stream at 40 °C.

Reconstitute with 200 μL dry toluene
Add 25 μL MTBSTFA
Cap and vortex mix for one minute

Incubate at 60° C for one hour

1.0 μL for injection

Figure 21: The optimized extraction scheme for DPMA and $[^{2}H_{10}]$DPMA from ovine plasma and urine.
3.1.2.3. Calibration Curve for DPMA and $[^2\text{H}_{10}]$DPMA

The calibration curves for DPMA and $[^2\text{H}_{10}]$DPMA were linear over the concentration range examined (i.e., 2.5 to 250.0 ng/mL). Weighted linear regression was carried out using the weighting function of $1/Y^2$ to reduce the bias of the interpolated concentrations at the low end of the assay. The resulting equations describe the linear regression for DPMA: $Y=0.0088X + 0.0009; r^2 = 1.000$, and $[^2\text{H}_{10}]$DPMA: $Y=0.0095X + (-0.0013); r^2 = 1.000$ (Figure 22). These regression constants resulted in a -9% bias at 2.5 ng/mL and +1% at 250.0 ng/mL for DPMA, and a +4% bias at 2.5 ng/mL and a +1% bias at 250.0 ng/mL for $[^2\text{H}_{10}]$DPMA. The minimum quantifiable concentration of this analytical method was 2.5 ng/mL (i.e., 11 pg at the detector), which corresponds to a signal-to-noise ratio of 15 for DPMA and 20 for $[^2\text{H}_{10}]$DPMA.

![Figure 22: Calibration curve for DPMA and $[^2\text{H}_{10}]$DPMA extracted from ovine plasma.](image)
3.1.2.4. Extractions Recovery of DPMA and $[^2\text{H}_{10}]$DPMA

The mean (± S.D.) extraction recoveries at various concentrations of DPMA and $[^2\text{H}_{10}]$DPMA from plasma were 78 ± 5% and 86 ± 11% at 5.0 ng/mL, 78 ± 1% and 75 ± 2% at 50.0 ng/mL, and 77 ± 2% and 74 ± 1% at 500.0 ng/mL, respectively. In urine, the extraction efficiency for DPMA and $[^2\text{H}_{10}]$DPMA was 95 ± 6% and 99 ± 11% at 5.0 ng/mL, 73 ± 9% and 74 ± 14% at 50.0 ng/mL, and 79 ± 2% and 74 ± 2% at 500.0 ng/mL, respectively.

3.1.2.5. Sample Stability Assessment

Plasma samples containing DPMA and $[^2\text{H}_{10}]$DPMA appeared to be stable when stored frozen for up to a period of 6 months. Following three freeze-thaw cycles, the concentration of DPMA and $[^2\text{H}_{10}]$DPMA did not differ significantly from the control values (Mann-Whitney U test; $P > 0.05$). Moreover, the slopes of the linear regression of the measured concentration of DPMA and $[^2\text{H}_{10}]$DPMA in plasma vs. time were not significantly different from zero (Two Sample T-test; $P > 0.05$) following bench-top storage for up to and including 24 hours. However, DPMA was labile when stored in an acidified sample matrix for a prolonged period of time. The calculated first order degradation half-life was 16.5 hours in water, 23.7 hours in blank plasma, and 33.6 hours in blank urine matrices (Figure 23). The area ratios of DPMA and $[^2\text{H}_{10}]$DPMA (i.e., DPMA/DPAA or $[^2\text{H}_{10}]$DPMA/DPAA) did not change for up to 96 hours of storage of prepared samples on the auto-sampler tray at room temperature (22°C).
Figure 23: Degradation of DPMA in acidified (0.4 mL of 1.0 M HCl) water, plasma and urine matrices (i.e., pH < 1.2).

3.2.1.6. Method Validation for DPMA and $^{[2H_{10}]}$DPMA Analysis

The results of the intra- and inter-day variability studies for this analytical method are shown in Table 3 and 4, respectively. The estimates of intra-day variability for DPMA and $^{[2H_{10}]}$DPMA were below 16% at the minimum quantifiable concentration of 2.5 ng/mL, and below 5% at all other concentrations investigated in plasma and urine (Table 3). The measured inter-day variability for DPMA and $^{[2H_{10}]}$DPMA was below 10% at the minimum quantifiable concentration of 2.5 ng/mL and below 8% for all other points (Table 4).
Table 3: Intra-day variability of DPMA/[^2H10]DPMA assay method in plasma and urine. Mean measured concentrations with standard deviation (SD) (n=4).

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Urine</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 ng/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>2.35</td>
<td>2.24</td>
<td>2.19</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.23</td>
<td>0.06</td>
<td>0.35</td>
</tr>
<tr>
<td>C.V. (%)</td>
<td>9.6</td>
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<td>15.9</td>
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<td>10.0 ng/mL</td>
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<tr>
<td>Mean</td>
<td>8.54</td>
<td>8.26</td>
<td>8.80</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.09</td>
<td>0.16</td>
<td>0.29</td>
</tr>
<tr>
<td>C.V. (%)</td>
<td>1.0</td>
<td>1.9</td>
<td>3.3</td>
</tr>
<tr>
<td>50.0 ng/mL</td>
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<td>Mean</td>
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<td>47.3</td>
</tr>
<tr>
<td>S.D.</td>
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<td>0.7</td>
<td>1.0</td>
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<td>C.V. (%)</td>
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<td>2.1</td>
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<td>250.0 ng/mL</td>
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<tr>
<td>Mean</td>
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<td>251.5</td>
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<tr>
<td>S.D.</td>
<td>2.2</td>
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<td>11.7</td>
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<tr>
<td>C.V. (%)</td>
<td>0.9</td>
<td>1.6</td>
<td>4.7</td>
</tr>
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</table>

Table 4: Inter-day variability of DPMA/[^2H10]DPMA assay method in plasma and urine. Mean measured concentrations with standard deviation (SD) (n=4).

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Urine</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 ng/mL</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>2.30</td>
<td>2.39</td>
<td>2.25</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.05</td>
<td>0.19</td>
<td>0.08</td>
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<tr>
<td>C.V. (%)</td>
<td>2.2</td>
<td>8.1</td>
<td>3.6</td>
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<tr>
<td>10.0 ng/mL</td>
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<tr>
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<td>8.93</td>
</tr>
<tr>
<td>S.D.</td>
<td>0.59</td>
<td>0.34</td>
<td>0.32</td>
</tr>
<tr>
<td>C.V. (%)</td>
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<tr>
<td>Mean</td>
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<td>45.5</td>
<td>47.3</td>
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<tr>
<td>S.D.</td>
<td>2.5</td>
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<tr>
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<td>6.3</td>
<td>0.3</td>
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</tr>
<tr>
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<td>250.8</td>
<td>247.8</td>
<td>253.0</td>
</tr>
<tr>
<td>S.D.</td>
<td>11.2</td>
<td>19.1</td>
<td>2.3</td>
</tr>
<tr>
<td>C.V. (%)</td>
<td>4.4</td>
<td>7.7</td>
<td>0.9</td>
</tr>
</tbody>
</table>
3.2. Animal Experimentation

The experimental details for all of the animal experiments conducted in this thesis are shown in Appendix 1

3.2.1. Disposition of DPHM in Non-Pregnant Sheep.

Initial experiments conducted to assess the renal contribution to the total body clearance of DPHM were carried out in five non-pregnant ewes with a mean (± SEM) weight of 73.7 ± 1.6 Kg (Appendix 1). Following a 100 mg IV bolus dose via the femoral vein of a non-pregnant sheep, the femoral arterial plasma concentrations of DPHM declined rapidly in a bi-exponential fashion. The mean (± SEM) femoral arterial plasma concentrations following drug administration are illustrated in figure 24. The mean total body clearance of DPHM in these non-pregnant sheep was 53.0 ± 10.4 mL/minute/Kg (Table 5). The mean (± SEM) cumulative renal excretion of the drug is shown in figure 25. The renal clearance, calculated by dividing the cumulative amount of DPHM excreted in the urine by the AUC may be an underestimation, since an accurate estimate of ΣXu is not always possible because urine was only collected for up to 12 hours in four of the five animals, and for 24 hours in the remaining ewe. In this latter animal, if urine collection had been stopped at 12 hours, ΣXu would have been underestimated by ~ 7%; therefore, the values of renal clearance presented in table 5 likely fall within a 10% error. The average renal clearance was 0.36 ± 0.28 mL/min/Kg, which represents approximately 0.3% of the total body clearance of the drug.
Bile was not collected continuously, thus, it is not possible to estimate by direct means the biliary secretion of DPHM. The concentrations of DPHM in bile following a 100 mg IV femoral venous bolus dose in E#248 and E#617 are shown in table 6.

Table 5: Pharmacokinetic Parameters following a 100 mg IV bolus of DPHM via the femoral vein in non-pregnant ewes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>E#543</th>
<th>E#248</th>
<th>E#316</th>
<th>E#105</th>
<th>E#617</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>α(min⁻¹)</td>
<td>0.095</td>
<td>0.590</td>
<td>0.182</td>
<td>0.033</td>
<td>0.040</td>
<td>0.22 ± 0.08</td>
</tr>
<tr>
<td>β(min⁻¹)</td>
<td>0.010</td>
<td>0.018</td>
<td>0.015</td>
<td>0.010</td>
<td>0.011</td>
<td>0.012 ± 0.001</td>
</tr>
<tr>
<td>α-T₁/₂ (min)</td>
<td>7.3</td>
<td>1.2</td>
<td>3.8</td>
<td>21.0</td>
<td>17.3</td>
<td>10.1 ± 3.9</td>
</tr>
<tr>
<td>β-T₁/₂ (min)</td>
<td>69.3</td>
<td>38.2</td>
<td>46.2</td>
<td>69.3</td>
<td>63.0</td>
<td>57.3 ± 6.3</td>
</tr>
<tr>
<td>CLₜ (mL/min/Kg)</td>
<td>35</td>
<td>70</td>
<td>87</td>
<td>35</td>
<td>38</td>
<td>53.0 ± 10.7</td>
</tr>
<tr>
<td>Vdₜₜ (L/Kg)</td>
<td>4.34</td>
<td>2.89</td>
<td>5.00</td>
<td>2.25</td>
<td>2.47</td>
<td>3.4 ± 0.5</td>
</tr>
<tr>
<td>MDRT (min)</td>
<td>123.9</td>
<td>41.0</td>
<td>57.5</td>
<td>65.1</td>
<td>64.8</td>
<td>70.5 ± 14.0</td>
</tr>
<tr>
<td>CLrenal (mL/min/Kg)</td>
<td>0.10</td>
<td>0.03</td>
<td>0.17</td>
<td>1.45</td>
<td>0.03</td>
<td>0.36 ± 0.28</td>
</tr>
<tr>
<td>% DOSE (Renal)</td>
<td>0.3</td>
<td>0.1</td>
<td>0.2</td>
<td>1.3</td>
<td>0.1</td>
<td>0.40 ± 0.23</td>
</tr>
</tbody>
</table>

Table 6: Biliary concentrations of DPHM obtained from periodic collections (i.e., 10 minute collections) of bile from two non-pregnant ewes following a 100 mg femoral venous bolus dose of DPHM.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>E#617</th>
<th>E#248</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[Bile] ng/mL</td>
<td>[Bile]/[Plasma]</td>
</tr>
<tr>
<td>15</td>
<td>922</td>
<td>1.7</td>
</tr>
<tr>
<td>30</td>
<td>492</td>
<td>1.6</td>
</tr>
<tr>
<td>45</td>
<td>302</td>
<td>1.3</td>
</tr>
<tr>
<td>60</td>
<td>190</td>
<td>1.0</td>
</tr>
<tr>
<td>120</td>
<td>25</td>
<td>0.4</td>
</tr>
<tr>
<td>180</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>
Figure 24: Mean femoral arterial plasma concentrations (± SEM) of DPHM following a 100 mg IV bolus \textit{via} the femoral vein in adult non-pregnant sheep (n=5).

Figure 25: The mean cumulative amount (± SEM) of DPHM excreted in the urine following a 100 mg IV bolus dose of DPHM \textit{via} the femoral vein in non-pregnant adult sheep (n=5).
3.2.2. **Isotope Effect Studies**

3.2.2.1. **Adult Non-Pregnant Sheep**

Control experiments were conducted to assess the presence of any possible isotope effects in the pharmacokinetic disposition of \([^{2}\text{H}_{10}]\text{DPHM}\). These experiments were conducted in 2 non-pregnant ewes. Figure 26 shows a representative plot of the femoral arterial plasma drug concentrations following equimolar doses of DPHM HCl and \([^{2}\text{H}_{10}]\text{DPHM HCl}\) equivalent to 100 mg of total DPHM free base \((i.e., \text{DPHM} + [^{2}\text{H}_{10}]\text{DPHM})\) in ewe 2169. The corresponding plot of the cumulative amount of DPHM and \([^{2}\text{H}_{10}]\text{DPHM}\) excreted in urine is shown in figure 27. The pharmacokinetic parameter estimates for the two animals are shown in table 7. In each animal there were no apparent differences between DPHM and \([^{2}\text{H}_{10}]\text{DPHM}\) arterial plasma concentrations and the pharmacokinetic estimates. In addition, the representative plot showing the plasma concentrations of the metabolites \((i.e., \text{DPMA} \ and \ [^{2}\text{H}_{10}]\text{DPMA})\) is shown for ewe 2169 in figure 28, with the corresponding plot of the cumulative amount of these metabolites excreted in urine in figure 29. Thus, it would seem that the pharmacokinetic equivalence of \([^{2}\text{H}_{10}]\text{DPHM}\) to DPHM also extends to this metabolic pathway. Overall, there do not appear to be differences between \([^{2}\text{H}_{10}]\text{DPHM}\) and DPHM in the parameters examined in this study.
Table 7: Pharmacokinetic parameters of DPHM and $[^{2}\text{H}_{10}]$DPHM following simultaneous IV bolus administration of equimolar amounts* of DPHM HCl and $[^{2}\text{H}_{10}]$DPHM HCl via the femoral vein in two non-pregnant ewes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>EWE 2167</th>
<th>EWE 2169</th>
<th>EWE 2167</th>
<th>EWE 2169</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$ (min$^{-1}$)</td>
<td>0.307</td>
<td>0.313</td>
<td>0.331</td>
<td>0.331</td>
</tr>
<tr>
<td>$\beta$ (min$^{-1}$)</td>
<td>0.022</td>
<td>0.022</td>
<td>0.044</td>
<td>0.044</td>
</tr>
<tr>
<td>$\alpha$ T$_{1/2}$ (min)</td>
<td>2.3</td>
<td>2.2</td>
<td>2.1</td>
<td>2.1</td>
</tr>
<tr>
<td>$\beta$ T$_{1/2}$ (min)</td>
<td>31.5</td>
<td>31.5</td>
<td>15.8</td>
<td>15.8</td>
</tr>
<tr>
<td>CL$_T$ (mL/min)</td>
<td>4000</td>
<td>3900</td>
<td>2700</td>
<td>2700</td>
</tr>
<tr>
<td>CL$_T$ (mL/min/Kg)</td>
<td>60</td>
<td>60</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Vd$_{ss}$ (L)</td>
<td>176.7</td>
<td>172.9</td>
<td>68.2</td>
<td>68.6</td>
</tr>
<tr>
<td>Vd$_{ss}$ (L/Kg)</td>
<td>2.49</td>
<td>2.44</td>
<td>0.97</td>
<td>0.98</td>
</tr>
<tr>
<td>Vd$_{h}$ (L)</td>
<td>186.5</td>
<td>179.6</td>
<td>61.8</td>
<td>62.1</td>
</tr>
<tr>
<td>Vd$_{h}$ (L/Kg)</td>
<td>2.63</td>
<td>2.53</td>
<td>0.88</td>
<td>0.89</td>
</tr>
<tr>
<td>MDRT min</td>
<td>43.6</td>
<td>44.1</td>
<td>24.3</td>
<td>24.9</td>
</tr>
<tr>
<td>AUC$_{0\rightarrow\infty}$ (ng*min/mL)</td>
<td>12499.6</td>
<td>12921.7</td>
<td>18252.8</td>
<td>18545.3</td>
</tr>
<tr>
<td>AUC$<em>{0\rightarrow\infty}$ (DPHM)/AUC$</em>{0\rightarrow\infty}$ ($[^{2}\text{H}_{10}]$DPHM)</td>
<td>0.97</td>
<td>1.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean $[Cp$ DPHM/Cp $[^{2}\text{H}_{10}]$DPHM]</td>
<td>0.99±0.04</td>
<td>0.98±0.07</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Total dose (i.e., DPHM + $[^{2}\text{H}_{10}]$DPHM) is equivalent to total dose of 100 mg DPHM free base. Weight corrected parameters based on the weights at the time of arrival to research facility.
Figure 26: A plot of the representative plasma concentrations of DPHM and $[^{2}\text{H}_{10}]$DPHM following an equimolar dose of DPHM HCl and $[^{2}\text{H}_{10}]$DPHM HCl administered simultaneously via the femoral vein to ewe 2169. (Isotope effect study).
Total dose equivalent to 100 mg of DPHM (i.e., DPHM + $[^{2}\text{H}_{10}]$DPHM) free base.
Figure 27: A representative plot of the cumulative amount of DPHM and $[^2\text{H}_{10}]$DPHM in urine following an equimolar dose of DPHM HCl and $[^2\text{H}_{10}]$DPHM HCl administered simultaneously via the femoral vein to ewe 2169. (Isotope effect study). Total dose equivalent to 100 mg of DPHM (i.e., DPHM + $[^2\text{H}_{10}]$DPHM) free base.
Figure 28: A representative plot of the plasma concentrations of DPMA and $[^{2}\text{H}_{10}]$DPMA following an equimolar dose of DPHM HCl and $[^{2}\text{H}_{10}]$DPHM HCl administered simultaneously via the femoral vein to ewe 2169. (Isotope effect study). Total dose equivalent to 100 mg of DPHM (i.e., DPHM + $[^{2}\text{H}_{10}]$DPHM) free base.
Figure 29: A representative plot of the cumulative amounts of DPMA and \([^{2}\text{H}_{10}]\)DPMA in urine following an equimolar dose of DPHM HCl and \([^{2}\text{H}_{10}]\)DPHM HCl administered simultaneously via the femoral vein to ewe 2169. (Isotope effect study). Total dose equivalent to 100 mg of DPHM (i.e., DPHM + \([^{2}\text{H}_{10}]\)DPHM) free base.
3.2.2.2. Fetal Lambs

3.2.2.2.1. Bolus Studies

Isotope effect studies were conducted on 5 fetal lambs following IV bolus administration (Appendix 1). The mean (± SEM) weight of the maternal sheep used in these experiments was 72.8 ± 3.7 Kg, while the mean fetal weight was 2.3 ± 0.3 Kg. The mean gestation age was 130 ± 1.8 days (Term 145 days). The total drug (DPHM + [2H10]DPHM) administered averaged 4.25 ± 0.93 mg/Kg of fetal body weight. The femoral arterial plasma concentrations of DPHM and [2H10]DPHM in a representative fetal lamb (E#975) following a simultaneous administration of equimolar amounts of DPHM and [2H10]DPHM are plotted in figure 30, while table 8 gives the pharmacokinetic parameters calculated for the two forms of the drug. No differences were apparent between the plasma concentrations of DPHM and [2H10]DPHM in each animal. In addition, there were no differences between the AUC0--∞ ratios for the labeled and unlabeled drug (Paired Sample T-test P>0.05). Further, no significant differences were noted between the pharmacokinetic parameters (i.e., α, β, CLT, Vdss, Vdb, and MDRT) of DPHM and [2H10]DPHM (Paired Sample T-test, P>0.05). A representative plot showing the concentrations of DPHM and [2H10]DPHM for E#975 in amniotic and fetal tracheal fluids is shown in figure 31. These data suggest that significant pharmacokinetic isotope effects do not exist for [2H10]DPHM in the fetal lamb following bolus administration over a 1 minute interval.
Table 8: Pharmacokinetic parameters of DPHM and $[^2\text{H}_{10}]$DPHM following simultaneous administration of equimolar amounts of DPHM HCl and $[^2\text{H}_{10}]$DPHM HCl via the fetal lateral tarsal vein (fetal isotope effect study).

<table>
<thead>
<tr>
<th>DPHM</th>
<th>$\alpha$ (min$^{-1}$)</th>
<th>$\beta$ (min$^{-1}$)</th>
<th>AUC (ng*min/mL)</th>
<th>CL$_\tau$ (mL/min/Kg)</th>
<th>Vd$\text{ss}$ (L/Kg)</th>
<th>Vd$\text{B}$ (L/Kg)</th>
<th>MDRT (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E#207</td>
<td>0.195</td>
<td>0.038</td>
<td>1514</td>
<td>1820</td>
<td>45</td>
<td>48</td>
<td>24.6</td>
</tr>
<tr>
<td>E#1143</td>
<td>0.196</td>
<td>0.043</td>
<td>6825</td>
<td>300</td>
<td>6</td>
<td>7</td>
<td>20.6</td>
</tr>
<tr>
<td>E#975</td>
<td>0.615</td>
<td>0.042</td>
<td>23091</td>
<td>100</td>
<td>3</td>
<td>3</td>
<td>25.1</td>
</tr>
<tr>
<td>E#102</td>
<td>0.092</td>
<td>0.035</td>
<td>1618</td>
<td>1100</td>
<td>32</td>
<td>31</td>
<td>30.1</td>
</tr>
<tr>
<td>E#1124</td>
<td>0.167</td>
<td>0.024</td>
<td>2275</td>
<td>600</td>
<td>24</td>
<td>27</td>
<td>35.9</td>
</tr>
</tbody>
</table>

| Mean   | 0.250                 | 0.036                | 7065            | 800                    | 22                   | 23                   | 27.3       |
| SEM    | 0.090                 | 0.004                | 4118            | 313                    | 8                    | 8                    | 2.6        |

<table>
<thead>
<tr>
<th>$[^2\text{H}_{10}]$DPHM</th>
</tr>
</thead>
<tbody>
<tr>
<td>E#207</td>
</tr>
<tr>
<td>E#1143</td>
</tr>
<tr>
<td>E#975</td>
</tr>
<tr>
<td>E#102</td>
</tr>
<tr>
<td>E#1124</td>
</tr>
</tbody>
</table>

| Mean   | 0.180                 | 0.036                | 7154            | 800                    | 23                   | 25                   | 27.6       |
| SEM    | 0.050                 | 0.003                | 4216            | 313                    | 8                    | 9                    | 3.0        |

Values are reported as mean ± SEM

*Weight corrected values are based on fetal body weight at the time of the experiment.
Figure 30: A representative figure of the arterial plasma concentration of DPHM and $[^2\text{H}_{10}]$DPHM following a simultaneous IV bolus dose of DPHM and $[^2\text{H}_{10}]$DPHM via the fetal lateral tarsal vein (E#975).
Figure 31: A representative plot of the amniotic and fetal tracheal fluid concentrations of DPHM and [²H₁₀]DPHM following simultaneous administration of DPHM and [²H₁₀]DPHM via the fetal lateral tarsal vein (E#975).
AMN - amniotic fluid and TR - fetal tracheal fluid
3.2.2.2 Infusion Study

Two experiments were conducted to examine the disposition of DPHM and $[^{2}\text{H}_{10}]$DPHM following simultaneous infusion of equimolar doses of DPHM and $[^{2}\text{H}_{10}]$DPHM (60 µg/minute of each DPHM and $[^{2}\text{H}_{10}]$DPHM) via the fetal lateral tarsal vein for 90 minutes. These experiments were conducted to check for differences in the disposition of the labeled drug in the fetal lamb following longer term drug administration. The concentrations of DPHM and $[^{2}\text{H}_{10}]$DPHM in fetal and maternal plasma, urine, and amniotic fluid, and, in addition, the plasma concentrations of DPMA and $[^{3}\text{H}_{10}]$DPMA were measured and are illustrated in figures 32-35, respectively. The mean steady-state concentrations of DPHM and $[^{2}\text{H}_{10}]$DPHM were 275.6 ± 16.2 and 272.8 ± 15.7 ng/mL for ewe 2241, and 95.5 ± 1.1 and 95.5 ± 1.8 ng/mL for ewe 2181, respectively (Figure 32). In addition, no apparent differences in the plasma concentrations of DPHM and $[^{2}\text{H}_{10}]$DPHM were observed in maternal plasma, amniotic fluid, and fetal urine (Figure 32-34). DPMA and $[^{2}\text{H}_{10}]$DPMA steady-state concentrations were not achieved during the course of the infusion. However, there were no apparent differences between the concentrations of the labeled and unlabeled form of the metabolite (Figure 35). Overall, the data do not indicate any significant isotope effect with labeled forms of DPHM and DPMA in the fetal lamb following a 90 minute infusion.
Figure 32: Plasma concentrations of DPHM and \(^{2}\text{H}_{10}\)DPHM in fetal and maternal plasma in ewes 2181 and 2241 following simultaneous infusions of 60 \(\mu\)g/min of each DPHM and \(^{2}\text{H}_{10}\)DPHM via the fetal lateral tarsal vein.

**Note:** fetal arterial plasma concentrations of DPHM and \(^{2}\text{H}_{10}\)DPHM are superimposed.
Figure 33: Amount of DPHM and $[^2\text{H}_{10}]$DPHM in fetal urine in ewes 2181 and 2241 following simultaneous infusions of 60 µg/min of each DPHM and $[^2\text{H}_{10}]$DPHM via the fetal lateral tarsal vein.

Figure 34: Concentrations of DPHM and $[^2\text{H}_{10}]$DPHM in amniotic fluid in ewes 2181 and 2241 following simultaneous infusions of 60 µg/min of each DPHM and $[^2\text{H}_{10}]$DPHM via the fetal lateral tarsal vein.
Figure 35: Concentrations of DPMA and $[^{2}\text{H}_{10}]$DPMA in fetal plasma in ewes 2181 and 2241 following simultaneous infusions of 60 μg/min of each DPHM and $[^{2}\text{H}_{10}]$DPHM via the fetal lateral tarsal vein.
3.2.3. Hepatic First-Pass Metabolism Studies

3.2.3.1. Non-Pregnant Sheep

3.2.3.1.1. Mesenteric Bolus Administration in Normoxic Conditions

The experimental details of this study are given in Appendix 1. The mean (± SEM) weight for the non-pregnant ewes used in first pass experiments was 70.2 ± 3.8 Kg. The mean total dose administered (DPHM + [²H₁₀]DPHM) was 1.64 ± 0.11 mg/Kg. The control (i.e., prior to experimentation) blood gas parameters were: pH, 7.43 ± 0.01; Po₂, 111 ± 8.6 mm Hg; and Pco₂, 41.3 ± 1.3 mm Hg, and experimental values were 7.43 ± 0.01, 107 ± 2.8 mm Hg, and 41.6 ± 0.1 mm Hg, respectively. There were no apparent differences in any of these parameters between the samples obtained during the control period and the samples obtained during the experiments.

In three of the ewes studied, [²H₁₀]DPHM was injected via the mesenteric vein and DPHM was simultaneously administered via the femoral vein, while in the other two animals the order was reversed to further minimize the likelihood of unmeasured isotope effects affecting our data (Appendix 1). A representative plot of the disposition of the two forms of the drug following the simultaneous administration of DPHM via the mesenteric vein (portal vein) and [²H₁₀]DPHM via the femoral vein (systemic) is shown in figure 36. The disposition of [²H₁₀]DPHM following administration via the femoral vein showed a bi-exponential decline. The apparent hepatic first-pass effect of DPHM was extensive since the plasma concentrations of DPHM following mesenteric venous administration were approximately ten fold lower than the plasma concentrations of the drug following femoral venous injection. Although the extraction following mesenteric venous administration was extensive, in most cases the plasma levels were
high enough to permit pharmacokinetic modeling, and thus, the calculation of the AUC$_{0-\infty}$.

However, in one animal (E#989), DPHM could be detected in the plasma following mesenteric administration (i.e., at 5 and 10 minutes post dose), but the plasma levels were too low to permit pharmacokinetic assessment. The pharmacokinetic parameters are shown in Table 9. Systemic bioavailability (F) averaged 0.068 (95% confidence interval: $0.026 \leq X \leq 0.111$) which corresponds to an extraction ratio ($E=1-F$) of $93.2 \pm 1.4\%$.

Table 9: Pharmacokinetic parameters obtained from non-pregnant sheep during a mesenteric first-pass metabolism study following the simultaneous administration of DPHM or [2H$_{10}$]DPHM via the femoral or mesenteric vein (n=5).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>E#139</th>
<th>E#1154</th>
<th>E#1158</th>
<th>E#989</th>
<th>E#102</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$ min$^{-1}$</td>
<td>0.103</td>
<td>0.331</td>
<td>0.196</td>
<td>0.200</td>
<td>0.181</td>
<td>0.20 ± 0.03</td>
</tr>
<tr>
<td>$\beta$ min$^{-1}$</td>
<td>0.039</td>
<td>0.044</td>
<td>0.023</td>
<td>0.023</td>
<td>0.049</td>
<td>0.036 ± 0.005</td>
</tr>
<tr>
<td>$\alpha$ T$_{1/2}$ min</td>
<td>6.7</td>
<td>2.1</td>
<td>3.5</td>
<td>3.5</td>
<td>3.8</td>
<td>3.9 ± 0.8</td>
</tr>
<tr>
<td>$\beta$ T$_{1/2}$ min</td>
<td>16.8</td>
<td>15.9</td>
<td>30.1</td>
<td>30.1</td>
<td>14.1</td>
<td>21.6 ± 3.5</td>
</tr>
<tr>
<td>CL$_T$ (mL/min/Kg)</td>
<td>58</td>
<td>55</td>
<td>46</td>
<td>80</td>
<td>87</td>
<td>65 ± 7</td>
</tr>
<tr>
<td>Vd$_{ss}$ (L) (L/Kg)</td>
<td>75.5 (1.2)</td>
<td>85.7 (1.4)</td>
<td>137.4 (2.1)</td>
<td>251.9 (2.9)</td>
<td>94.0 (1.3)</td>
<td>129 ± 29 (1.8 ± 0.3)</td>
</tr>
<tr>
<td>Vd$_{8}$ (L) (L/Kg)</td>
<td>91.9 (1.5)</td>
<td>110.9 (1.8)</td>
<td>128.8 (2.0)</td>
<td>307.6 (3.5)</td>
<td>128.7 (1.8)</td>
<td>153.6 ± 78.2 (2.1 ± 0.3)</td>
</tr>
<tr>
<td>MDRT (min)</td>
<td>20.4</td>
<td>24.3</td>
<td>45.7</td>
<td>35.9</td>
<td>14.4</td>
<td>28.1 ± 5.0</td>
</tr>
<tr>
<td>F</td>
<td>0.042</td>
<td>0.095</td>
<td>0.096</td>
<td>N/A</td>
<td>0.040</td>
<td>0.068 ± 0.014</td>
</tr>
</tbody>
</table>

**Pharmacokinetic parameters are calculated from the drug species administered via the femoral vein (i.e., DPHM or [2H$_{10}$]DPHM).**

N/A - plasma levels following the mesenteric dose were too low to permit pharmacokinetic analysis.

The weight corrected parameters are based on the weights at the time of arrival of the sheep at the research facility.
3.3.3.1.2. Mesenteric Bolus Administration during Mild Hypoxemia

The control (i.e., prior to experimentation) blood gas parameters measured in the non-pregnant animals were: pH, 7.43 ± 0.02; Po2, 123 ± 5.0 mm Hg; O2 saturation 100.9 ± 0.5%; and Pco2, 38.7 ± 1.2 mm Hg, and experimental values were 7.44 ± 0.01, 62.4 ± 1.9 mm Hg, 82.9 ± 1.1%, and 38.3 ± 1.0 mm Hg, respectively. Statistically significant differences were noted between control and test period for Po2 and O2 saturation (p<0.05 Mann Whitney U -Test).

Although the mean reduction in the Po2 and O2 saturation appeared to be similar for all animals, the initial (i.e., first 30 minutes following drug administration) reduction in these parameters was greatest in ewes 1154 and 1158 (Table 10).

Figure 36: A representative plot of the plasma concentrations of DPHM and \([H_{10}]\)DPHM in a non-pregnant ewe (E#1154) following simultaneous mesenteric (DPHM) and femoral venous (\([H_{10}]\)DPHM) administration.
Table 10: Initial (i.e., the first 30 minutes) and average percentage changes in blood gas status following the initiation of mild to moderate hypoxemia induced by nitrogen gas infusion into the trachea of non-pregnant sheep.

<table>
<thead>
<tr>
<th>EWE</th>
<th>% Change Pco₂</th>
<th>% Change pH</th>
<th>% Change Po₂</th>
<th>% Change O₂ Saturation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial Mean (SEM)</td>
<td>Initial Mean (SEM)</td>
<td>Initial Mean (SEM)</td>
<td>Initial Mean (SEM)</td>
</tr>
<tr>
<td>E#102</td>
<td>-3.7 (3.8)</td>
<td>-0.27 (0.2)</td>
<td>-39.5 (7.6)</td>
<td>-12.3 (8.2)</td>
</tr>
<tr>
<td>E#139</td>
<td>-0.3 (2.0)</td>
<td>-0.68 (0.1)</td>
<td>-37.9 (4.2)</td>
<td>-12.3 (8.2)</td>
</tr>
<tr>
<td>E#989</td>
<td>-12.4 (7.0)</td>
<td>0.27 (0.3)</td>
<td>-45.2 (5.1)</td>
<td>-7.7 (5.9)</td>
</tr>
<tr>
<td>E#1154</td>
<td>2.9 (9.9)</td>
<td>0.53 (0.5)</td>
<td>-63.2 (3.7)</td>
<td>-30.9 (4.6)</td>
</tr>
<tr>
<td>E#1158</td>
<td>8.3 (1.7)</td>
<td>0.26 (0.2)</td>
<td>-58.8 (3.3)</td>
<td>-25.6 (2.9)</td>
</tr>
</tbody>
</table>

The pharmacokinetic parameters calculated for the animals during hypoxemia are shown in Table 11. There are no statistically significant differences between the pharmacokinetic parameters calculated during the normoxic and hypoxemic periods (Mann-Whitney U test, P>0.05). Only in two animals was it possible to calculate the bioavailability, because in three of the animals the plasma concentrations of the drug species administered via the mesenteric vein were too low to permit pharmacokinetic assessment. In the other two animals, namely, ewes 1154 and 1158, a larger bioavailability was calculated (Table 11). However, these estimates are likely to be inaccurate due to the difficulty of obtaining terminal elimination rate constants, and estimating the initial plasma concentrations (See figure 37).
Figure 37: Representative plots of the plasma concentrations of DPHM and $[^{2}H_{10}]$DPHM in non-pregnant ewes (E#1158-above) and (E#102-below) following simultaneous mesenteric (DPHM) and femoral venous ($[^{2}H_{10}]$DPHM) administration during mild hypoxemia.
Table 11: Pharmacokinetic parameters obtained from the non-pregnant mesenteric first-pass metabolism study following the simultaneous administration of DPHM or $[^2\text{H}_{10}]$DPHM via the femoral or mesenteric vein during mild hypoxemia (n=5).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>E#139</th>
<th>E#1154</th>
<th>E#1158</th>
<th>E#989</th>
<th>E#102</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha \text{ min}^{-1}$</td>
<td>0.2505</td>
<td>0.128</td>
<td>0.996</td>
<td>0.190</td>
<td>0.277</td>
<td>0.37 ± 0.16</td>
</tr>
<tr>
<td>$\beta \text{ min}^{-1}$</td>
<td>0.068</td>
<td>0.023</td>
<td>0.032</td>
<td>0.026</td>
<td>0.046</td>
<td>0.039 ± 0.009</td>
</tr>
<tr>
<td>$CL_T \text{ (mL/min/Kg)}$</td>
<td>67</td>
<td>58</td>
<td>219</td>
<td>87</td>
<td>123</td>
<td>111 ± 29</td>
</tr>
<tr>
<td>$V_d_{ss} \text{ (L/L/Kg)}$</td>
<td>97.9 (1.56)</td>
<td>85.9 (1.36)</td>
<td>244.0 (3.74)</td>
<td>237.8 (2.74)</td>
<td>160.7 (2.23)</td>
<td>165 ± 33 (2.33 ± 0.43)</td>
</tr>
<tr>
<td>$V_d_{b} \text{ (L/L/Kg)}$</td>
<td>91.9 (1.47)</td>
<td>110.9 (1.76)</td>
<td>128.8 (1.97)</td>
<td>307.6 (3.51)</td>
<td>128.7 (1.79)</td>
<td>154 ± 35 (2.10 ± 0.32)</td>
</tr>
<tr>
<td>MDRT (min)</td>
<td>23.4</td>
<td>23.5</td>
<td>17.1</td>
<td>31.7</td>
<td>18.1</td>
<td>22.8 ± 2.6</td>
</tr>
<tr>
<td>$F$</td>
<td>N/A</td>
<td>0.69</td>
<td>2.15</td>
<td>N/A</td>
<td>N/A</td>
<td>-</td>
</tr>
</tbody>
</table>

**pharmacokinetic parameters are calculated from the drug species administered via the femoral vein (i.e., DPHM or $[^2\text{H}_{10}]$DPHM).
N/A - plasma levels of mesenteric dose were too low to permit pharmacokinetic analysis.
The weight corrected parameters are based on the weights of the sheep at the time of arrival at the research facility.

3.2.3.2. Fetal Lambs

The mean maternal weight (± SEM) of the pregnant ewes used in the fetal umbilical venous first-pass experiments was 79.4 ± 4.9 Kg, and the mean fetal weight was 2.3 ± 0.3 Kg. The mean maternal weight of the pregnant ewes used in the simultaneous umbilical/tarsal venous infusion study was 74.5 ± 0.7 Kg, while the fetal weights were 2.0 ± 0.4 Kg. The mean fetal gestational age was 130 ± 1.8 days and 130 ± 2.7 days (term 145 days) for the fetal first-pass bolus and infusion experiments, respectively. In some cases, pregnant animals were used in both control (isotope effect studies) and first-pass experiments (Appendix 1). A minimum wash-out period of 48 hours was allowed prior to a second experiment. In fetal lambs, prior to experimentation, the measured blood gas values were: pH 7.31 ± 0.02; Po2 22.3 ± 1.7 mm Hg; Pco2 47.8 ± 0.9 mm Hg; O2 saturation 57.3 ± 5.9%, and hemoglobin concentration 10.5 ± 0.8 g/dL. During experimentation these values were 7.32 ± 0.02, 23.1 ± 1.4 mm Hg, 48.1 ± 1.1 mm
Hg, O₂ saturation 54.2 ± 4.9%, and hemoglobin concentration 10.3 ± 0.8 g/dL. The experimental blood gas values remained unchanged from control values during the experiment.

3.2.3.2.1. Umbilical Venous Bolus Administration

In the fetal experiment involving simultaneous drug injection via the umbilical and lateral tarsal veins, the combined drug dose (DPHM + [²H₁₀]DPHM) averaged 3.80 ± 0.6 mg/Kg (n=6). In three of the experiments [²H₁₀]DPHM was administered via the umbilical vein and DPHM via the lateral tarsal vein, while in the other three fetal lambs, the order of injection was reversed. A representative example of the arterial plasma drug concentration vs. time curve for one fetus (E#499) is illustrated in figure 38, while the pharmacokinetic parameters calculated using both femoral and carotid arterial plasma drug concentrations are given in table 12 and 13, respectively. The plasma concentrations of DPHM administered via the umbilical vein (test) were similar to those observed following tarsal venous administration (control). Thus, in contrast to the situation in adult sheep, a fetal hepatic first-pass effect following umbilical venous bolus administration was not apparent. The systemic availability (F) of DPHM measured using femoral arterial blood following umbilical administration was 1.10 ± 0.08 (95% confidence interval 0.9<X<1.3), corresponding to an extraction (E) of -10 ± 8 %. There were no statistically significant differences between the pharmacokinetic parameters calculated using either the femoral or the carotid arterial reference sites (Paired Sample T-test, P>0.05). The availability measured from carotid arterial blood was 1.20 ± 0.11, and was not different from the value measured using femoral arterial blood samples (Paired Sample T-test p>0.05).
Table 12: Pharmacokinetic parameters calculated using femoral arterial plasma drug concentrations during fetal umbilical first-pass metabolism experiments following the simultaneous administration of DPHM and [2H10]DPHM.

### Tarsal Venous Administration - Femoral Arterial Reference

<table>
<thead>
<tr>
<th>Parameter</th>
<th>E#989</th>
<th>E#208</th>
<th>E#499</th>
<th>E#1143</th>
<th>E#543</th>
<th>E#975</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$ min$^{-1}$</td>
<td>0.149</td>
<td>0.415</td>
<td>0.238</td>
<td>0.225</td>
<td>0.267</td>
<td>0.139</td>
<td>0.24 ± 0.04</td>
</tr>
<tr>
<td>$\beta$ min$^{-1}$</td>
<td>0.030</td>
<td>0.023</td>
<td>0.029</td>
<td>0.039</td>
<td>0.025</td>
<td>0.030</td>
<td>0.029 ± 0.003</td>
</tr>
<tr>
<td>AUC (ng*min/mL)</td>
<td>2436</td>
<td>2045</td>
<td>2828</td>
<td>2453</td>
<td>1636</td>
<td>10881</td>
<td>3713 ± 1579</td>
</tr>
<tr>
<td>CLT$_T$ (mL/min/Kg)</td>
<td>387</td>
<td>647</td>
<td>816</td>
<td>1063</td>
<td>879</td>
<td>263</td>
<td>700 ± 100</td>
</tr>
<tr>
<td>$V_d$ss L (L/Kg)</td>
<td>35.5 (13.6)</td>
<td>45.7 (24.8)</td>
<td>60.4 (27.4)</td>
<td>48.9 (26.1)</td>
<td>115.9 (33.9)</td>
<td>14.2 (8.1)</td>
<td>53 ± 15.2 (22 ± 4.5)</td>
</tr>
<tr>
<td>$V_d$B L (L/Kg)</td>
<td>33.7 (12.9)</td>
<td>52.9 (28.7)</td>
<td>62.5 (28.4)</td>
<td>51.6 (27.5)</td>
<td>122 (35.6)</td>
<td>15.5 (8.8)</td>
<td>56 ± 16.1 (24 ± 4.5)</td>
</tr>
<tr>
<td>MDRT (min)</td>
<td>34.7</td>
<td>37.8</td>
<td>33.1</td>
<td>24</td>
<td>38</td>
<td>30.2</td>
<td>33 ± 22</td>
</tr>
<tr>
<td>F</td>
<td>0.91</td>
<td>0.93</td>
<td>1.27</td>
<td>1.36</td>
<td>0.95</td>
<td>1.15</td>
<td>1.10 ± 0.08</td>
</tr>
</tbody>
</table>

### Umbilical Venous Administration - Femoral Arterial Reference

<table>
<thead>
<tr>
<th>Parameter</th>
<th>E#989</th>
<th>E#208</th>
<th>E#499</th>
<th>E#1143</th>
<th>E#543</th>
<th>E#975</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$ min$^{-1}$</td>
<td>0.163</td>
<td>0.253</td>
<td>0.281</td>
<td>0.437</td>
<td>0.254</td>
<td>0.131</td>
<td>0.25 ± 0.5</td>
</tr>
<tr>
<td>$\beta$ min$^{-1}$</td>
<td>0.032</td>
<td>0.022</td>
<td>0.029</td>
<td>0.040</td>
<td>0.025</td>
<td>0.029</td>
<td>0.030 ± 0.003</td>
</tr>
<tr>
<td>AUC (ng*min/mL)</td>
<td>2226</td>
<td>1907</td>
<td>3605</td>
<td>3335</td>
<td>1549</td>
<td>12465</td>
<td>4257 ± 1892</td>
</tr>
<tr>
<td>CLT$_T$ (mL/min/Kg)</td>
<td>424</td>
<td>695</td>
<td>640</td>
<td>782</td>
<td>929</td>
<td>229</td>
<td>600 ± 130</td>
</tr>
<tr>
<td>$V_d$ss L (L/Kg)</td>
<td>36.8 (14.1)</td>
<td>54.1 (29.3)</td>
<td>41.9 (19)</td>
<td>33.7 (18)</td>
<td>123 (35.9)</td>
<td>10.9 (6.2)</td>
<td>50 ± 17 (20 ± 9.9)</td>
</tr>
<tr>
<td>$V_d$B L (L/Kg)</td>
<td>34.1 (13.1)</td>
<td>57.7 (31.3)</td>
<td>48.6 (22.1)</td>
<td>36.4 (19.4)</td>
<td>125.5 (36.6)</td>
<td>13.8 (7.8)</td>
<td>53 ± 17.4 (22 ± 4.9)</td>
</tr>
<tr>
<td>MDRT (min)</td>
<td>32.8</td>
<td>41.7</td>
<td>29.2</td>
<td>22.5</td>
<td>38.2</td>
<td>26.5</td>
<td>32 ± 3.1</td>
</tr>
</tbody>
</table>

*Weight corrected values are based on fetal body weight at the time of the experiment. There were no statistical differences between the pharmacokinetic parameters calculated from the femoral arterial and carotid arterial reference sites (p>0.05 paired sample T-test).
Table 13: Pharmacokinetic parameters calculated using carotid arterial plasma drug concentrations during fetal umbilical first-pass metabolism experiments following the simultaneous administration of DPHM and $[^2H_{10}]$DPHM.

Tarsal Venous Administration - Carotid Arterial Reference

<table>
<thead>
<tr>
<th>Parameter</th>
<th>E#989</th>
<th>E#208</th>
<th>E#499</th>
<th>E#1143</th>
<th>E#543</th>
<th>E#975</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha \text{ min}^{-1}$</td>
<td>0.2122</td>
<td>0.7935</td>
<td>0.2183</td>
<td>0.852</td>
<td>0.0778</td>
<td>0.1744</td>
<td>0.39 ± 0.15</td>
</tr>
<tr>
<td>$\beta \text{ min}^{-1}$</td>
<td>0.0363</td>
<td>0.0249</td>
<td>0.0330</td>
<td>0.0406</td>
<td>0.0198</td>
<td>0.0325</td>
<td>0.031 ± 0.004</td>
</tr>
<tr>
<td>AUC (mL/min/Kg)</td>
<td>2166</td>
<td>2827</td>
<td>2746</td>
<td>1827</td>
<td>1419</td>
<td>8998</td>
<td>3388 ± 1291</td>
</tr>
<tr>
<td>$CL_T$ (mL/min/Kg)</td>
<td>436</td>
<td>469</td>
<td>841</td>
<td>1428</td>
<td>1014</td>
<td>318</td>
<td>800 ± 200</td>
</tr>
<tr>
<td>$V_{ds}$ L (L/Kg)</td>
<td>33.2</td>
<td>38.4</td>
<td>54.4</td>
<td>69</td>
<td>140.2</td>
<td>16.6</td>
<td>59 ± 19.6</td>
</tr>
<tr>
<td>$V_d$ L (L/Kg)</td>
<td>31.2</td>
<td>34.8</td>
<td>56</td>
<td>65.9</td>
<td>175.6</td>
<td>1.7</td>
<td>61 ± 26.8</td>
</tr>
<tr>
<td>MDRT (min)</td>
<td>28.7</td>
<td>43.9</td>
<td>28.9</td>
<td>25.2</td>
<td>39.9</td>
<td>29.2</td>
<td>33 ± 3.1</td>
</tr>
<tr>
<td>$F$</td>
<td>0.98</td>
<td>1.01</td>
<td>1.54</td>
<td>1.44</td>
<td>1.18</td>
<td>1.03</td>
<td>1.20 ± 0.10</td>
</tr>
</tbody>
</table>

Umbilical Venous Administration - Carotid Arterial Reference.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>E#989</th>
<th>E#208</th>
<th>E#499</th>
<th>E#1143</th>
<th>E#543</th>
<th>E#975</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha \text{ min}^{-1}$</td>
<td>0.053</td>
<td>0.8485</td>
<td>0.3719</td>
<td>0.2336</td>
<td>0.1302</td>
<td>0.3023</td>
<td>0.32 ± 0.13</td>
</tr>
<tr>
<td>$\beta \text{ min}^{-1}$</td>
<td>0.0221</td>
<td>0.0230</td>
<td>0.0321</td>
<td>0.0351</td>
<td>0.0175</td>
<td>0.0332</td>
<td>0.027 ± 0.003</td>
</tr>
<tr>
<td>AUC (mL/min/Kg)</td>
<td>2128</td>
<td>2804</td>
<td>4236</td>
<td>2622</td>
<td>1675</td>
<td>9254</td>
<td>3786 ± 1257</td>
</tr>
<tr>
<td>$CL_T$ (mL/min/Kg)</td>
<td>443</td>
<td>472</td>
<td>545</td>
<td>995</td>
<td>859</td>
<td>309</td>
<td>600 ± 130</td>
</tr>
<tr>
<td>$V_{ds}$ L (L/Kg)</td>
<td>29.7</td>
<td>36.7</td>
<td>26.7</td>
<td>47.6</td>
<td>97.2</td>
<td>15.1</td>
<td>42 ± 12.4</td>
</tr>
<tr>
<td>$V_d$ L (L/Kg)</td>
<td>52.3</td>
<td>38</td>
<td>37.4</td>
<td>53.1</td>
<td>167.9</td>
<td>16.3</td>
<td>61 ± 24.1</td>
</tr>
<tr>
<td>MDRT (min)</td>
<td>25.2</td>
<td>41.6</td>
<td>21.7</td>
<td>25</td>
<td>32.6</td>
<td>27.3</td>
<td>29 ± 3.1</td>
</tr>
</tbody>
</table>

*Weight corrected values are based on fetal body weight at the time of the experiment.

There were no statistical differences between the pharmacokinetic parameters calculated from the femoral arterial and carotid arterial reference sites (p>0.05 paired sample T-test)
3.2.3.2.3. Umbilical Venous Infusions

Fetal hepatic extraction was investigated with simultaneous 90 minute infusions of DPHM and $[^2H_{10}]$DPHM via the umbilical and fetal lateral tarsal veins. The mean (± SEM) infusion rate for both DPHM and $[^2H_{10}]$DPHM was $57.2 ± 10.8 \mu g/min$ of each. The mean concentrations of drug in the femoral and carotid arterial plasma following umbilical venous infusion were $64.8 ± 13.2 \text{ ng/mL}$ and $61.3 ± 11.9 \text{ ng/mL}$, respectively. The femoral and carotid arterial plasma concentrations following tarsal venous infusion were $66.6 ± 13.1$ and $59.6 ± 11.9$
ng/mL. The total body clearances (CL_T) measured from carotid and femoral arterial samples for both routes of administration are shown in table 14. The differences between the total body clearances following tarsal venous and umbilical venous administration were 28.6 ± 44.8 mL/min/Kg, and -20.2 ± 53.9 mL/min/Kg measured from femoral and carotid arterial blood, respectively. Although the net hepatic clearance appeared to decrease when measured from carotid arterial blood as compared to femoral arterial blood, no statistically significant differences were noted (Table 14). During the 90 minute infusion period, there were statistically significant differences between the femoral and carotid arterial plasma concentrations of lactate and glucose, oxygen saturation, and the partial pressure of oxygen (Table 15). All of these parameters were higher in carotid arterial blood compared to the femoral arterial blood (See Table 15). In contrast, the concentrations of both forms of the drug where higher in FA than in CA (Table 15). The mean differences were 8.5 ± 2.1 ng/mL and 3.3 ± 1.3 ng/mL, for tarsal and umbilical venous drug infusion, respectively. Both mean differences were statistically different from zero (2 Sample T-test, p<0.05). Moreover, the difference measured following umbilical venous administration was lower than that measured following tarsal venous infusion.
Table 14: Clearances calculated following simultaneous steady-state umbilical and tarsal venous infusions of DPHM or $[^{2}H_{10}]$DPHM

<table>
<thead>
<tr>
<th>EWE</th>
<th>$CL_{tv}$ (FA) (mL/min/Kg)</th>
<th>$CL_{uv}$ (FA) (mL/min/Kg)</th>
<th>$CL_{tv}$ (CA) (mL/min/Kg)</th>
<th>$CL_{uv}$ (CA) (mL/min/Kg)</th>
<th>$CL_{uv}$-$CL_{tv}$ (FA)</th>
<th>$CL_{uv}$-$CL_{tv}$ (CA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E#1142</td>
<td>447</td>
<td>645</td>
<td>582</td>
<td>691</td>
<td>191</td>
<td>118</td>
</tr>
<tr>
<td>E#1242(2)</td>
<td>650</td>
<td>659</td>
<td>608</td>
<td>593</td>
<td>18.6</td>
<td>-3.8</td>
</tr>
<tr>
<td>E#1242(1)</td>
<td>303</td>
<td>349</td>
<td>330</td>
<td>373</td>
<td>38.8</td>
<td>39.2</td>
</tr>
<tr>
<td>E#1250</td>
<td>468</td>
<td>407</td>
<td>520</td>
<td>472</td>
<td>-61</td>
<td>-48</td>
</tr>
<tr>
<td>E#2164</td>
<td>1158</td>
<td>1113</td>
<td>1341</td>
<td>1135</td>
<td>-44</td>
<td>-21</td>
</tr>
<tr>
<td>Mean±SEM</td>
<td>605±149</td>
<td>634±135</td>
<td>676±173</td>
<td>653±132</td>
<td>28.6±44.8</td>
<td>-20.2±53.9</td>
</tr>
</tbody>
</table>

* The difference is taken at each time sampling time point (i.e., 15, 30, 45, 60, 75, and 90 minutes for each animal). These do not represent the difference of the mean values.
TV and UV represent the route of administration (i.e., tarsal vein or umbilical vein) of DPHM or $[^{2}H_{10}]$DPHM.

Table 15: Blood gas parameters during simultaneous umbilical venous and tarsal venous infusion of DPHM or $[^{2}H_{10}]$DPHM to steady-state (n=5).

<table>
<thead>
<tr>
<th></th>
<th>Femoral artery</th>
<th>Carotid artery</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.348±0.008</td>
<td>7.356±0.008</td>
</tr>
<tr>
<td>$P_{CO_2}$ (mm Hg)</td>
<td>48.5±1.0</td>
<td>47.4±1.0*</td>
</tr>
<tr>
<td>$P_{O_2}$ (mm Hg)</td>
<td>20.3±1.3</td>
<td>22.9±1.4***</td>
</tr>
<tr>
<td>Base Excess (meq/L)</td>
<td>1.5±0.5</td>
<td>1.6±0.3</td>
</tr>
<tr>
<td>$HCO_3$ (meq/L)</td>
<td>26.3±0.5</td>
<td>26.1±0.4</td>
</tr>
<tr>
<td>TCO$_2$</td>
<td>27.6±0.5</td>
<td>27.4±0.4</td>
</tr>
<tr>
<td>Hemoglobin (g/dL)</td>
<td>9.7±0.8</td>
<td>9.6±0.8</td>
</tr>
<tr>
<td>$O_2$ Saturation (%v/v)</td>
<td>47.2±5.8</td>
<td>54.5±8.0***</td>
</tr>
<tr>
<td>[Lactate] mmol</td>
<td>1.4±0.1</td>
<td>1.5±0.1**</td>
</tr>
<tr>
<td>[Glucose] mmol</td>
<td>0.9±0.3</td>
<td>1.1±0.5**</td>
</tr>
<tr>
<td>[DPHM] uv (ng/mL)</td>
<td>64.8±13.2</td>
<td>61.3±11.9*</td>
</tr>
<tr>
<td>[DPHM] tv (ng/mL)</td>
<td>66.6±13.1</td>
<td>59.6±11.9*</td>
</tr>
</tbody>
</table>

Data is presented as mean ± SEM
* statistically significant difference p<0.05 [paired sample T-test]
** statistically significant difference p<0.02 [paired sample T-test]
*** statistically significant difference p<0.002 [paired sample T-test]
3.2.4. Paired Fetal-Maternal Infusions

The five experiments involving simultaneous 6 hour infusions of DPHM and $[^{2}\text{H}_{10}]$DPHM to ewe and fetus, respectively, were carried out at 125-133 days gestation age (129 ± 1 days). Estimated fetal weight was $2.24 \pm 0.06$ Kg. In the control period the fetal descending aortic values for pH, $\text{P}O_2$, $\text{P}co_2$, $\text{O}_2$ saturation, hemoglobin concentration, glucose concentration, and lactate concentration were $7.36 \pm 0.02$, $22.6 \pm 1.65$ mm Hg, $47.3 \pm 0.5$ mm Hg, $55.3 \pm 23.8$ %, $10.0 \pm 0.3$ g/dL, $0.98 \pm 0.09$ mM., and $0.70 \pm 0.11$ mM.

3.2.4.1. Estimates of Trans-Placental and Non-Placental Clearances

The average (± SEM) fetal and maternal femoral arterial plasma concentrations of DPHM and $[^{2}\text{H}_{10}]$DPHM in maternal and fetal plasma in five experiments involving 6 hour paired maternal (DPHM - 670 µg/min) and fetal (170 µg/min - $[^{2}\text{H}_{10}]$DPHM) infusions are illustrated in figure 39. The plasma concentrations reach a plateau at approximately 150 minutes. There were no statistical differences between the plasma concentrations at 150 and 180, 240 and 270, and 330 and 360 minutes (p>0.05 repeated measures ANOVA), suggesting that steady-state was achieved by 150 minutes. Thus, the mean steady-state concentrations were calculated from 150 to 360 minutes, and are shown in table 16. The mean steady-state concentrations (± SEM) of DPHM were $262.0 \pm 24.2$, $27.2 \pm 4.4$, and $26.6 \pm 4.3$ ng/mL in the maternal femoral (MA), fetal femoral (FA), and fetal carotid (CA) arteries, respectively. The mean concentrations of $[^{2}\text{H}_{10}]$DPHM were $44.9 \pm 7.4$, $203.7 \pm 29.9$, $186.1 \pm 27.0$ ng/mL in the MA, FA, and CA, respectively. The above mean values were calculated excluding ewe 2181, since CA samples were not available for this animal (Table 16). The total FA, CA, and MA steady-state concentrations of DPHM (i.e., DPHM + $[^{2}\text{H}_{10}]$DPHM) were $282.4 \pm 57.4$ ng/mL [231.0 ± 31.5


ng/mL, excluding ewe 2181) (FA), 212.8 ± 25.9 ng/mL (CA), and 308.1 ± 25.8 ng/mL (maternal). There were statistically significant differences between the FA and CA plasma concentrations of $[^{2}H_{10}]$DPHM (Paired Sample T-test, $p<0.05$). Although the mean concentrations of DPHM appeared higher in FA compared to CA blood (Table 16), these differences were not statistically significant for DPHM (maternally derived) and total DPHM (labeled + unlabeled) (Paired Sample T-test $p>0.05$). Following the cessation of the infusion, concentrations of DPHM and $[^{2}H_{10}]$DPHM in fetal and maternal plasma declined rapidly, with an apparent terminal elimination half-life in plasma of $77.5 ± 3.2$ minutes and $52.3 ± 7.0$ minutes in mother and fetus, respectively (Table 17). Both the corrected (i.e., corrected for loss of drug from fetus to mother or mother to fetus via the placenta) and the uncorrected volumes of distribution were larger in the fetus as compared to the mother (i.e., $27.1 ± 5.9$ L/Kg and $9.2 ± 1.7$ L/Kg vs. $2.0 ± 0.24$ L/Kg and $1.9 ± 0.23$ L/Kg) (Table 18). As in previous studies, there was accumulation of DPHM (both labeled and unlabeled) in fetal lung and amniotic fluids (Figure 40). The average drug concentration ratio between lung fluid and femoral arterial plasma was $(4.0 ± 1.7)$ for DPHM and $(4.5 ± 1.6)$ for $[^{2}H_{10}]$DPHM. During the infusion, the corresponding ratios in amniotic fluid (i.e., amniotic fluid/FA) were $0.6 ± 0.2$ and $0.8 ± 0.2$ for labeled and unlabeled drug, respectively. Although the ratio is lower in amniotic fluid, the drug persists longer in amniotic fluid than in fetal lung fluid.

The non- and trans-placental clearance parameters calculated using fetal and maternal arterial plasma drug concentrations are shown in Table 19. The weight normalized estimates of CLfm ($221 ± 32$ mL/min/Kg) and CLfo ($135 ± 26$ mL/min/Kg) were both significantly higher than the corresponding maternal values (CLmf $53.7 ± 14.4$ mL/min/Kg and CLmo $38 ± 2$ mL/min/Kg) (Paired Sample T-test $p<0.05$). The non-placental contribution to total body
clearance averaged (95.6 ± 1.0%) and (37.3 ± 4.9%) in ewe and fetus, respectively. The non- and trans-placental clearances were also calculated using a mass balance approach (Table 20). These values are in good agreement with those calculated using the approach of Szeto et al. (1982) (Table 19).

Table 16: Mean (± SEM) maternal and fetal steady-state arterial plasma drug concentrations following simultaneous maternal/fetal infusions.

<table>
<thead>
<tr>
<th>Reference Site</th>
<th>E#122Z (ng/mL)</th>
<th>E#2101 (ng/mL)</th>
<th>E#2177 (ng/mL)</th>
<th>E#2181 (ng/mL)</th>
<th>E#2241 (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA (DPHM)</td>
<td>27.2 (1.2)</td>
<td>14.8 (3.2)</td>
<td>31.9 (1.7)</td>
<td>111.2 (5.6)</td>
<td>34.9 (1.6)</td>
</tr>
<tr>
<td>FA ([2H10]DPHM)</td>
<td>137.5 (2.4)</td>
<td>207.4 (29.7)</td>
<td>187.9 (6.9)</td>
<td>378.3 (10.2)</td>
<td>281.8 (8.9)</td>
</tr>
<tr>
<td>CA (DPHM)</td>
<td>25.1 (1.4)</td>
<td>15.3 (2.6)</td>
<td>34.7 (1.9)</td>
<td>N/A</td>
<td>31.4 (2.6)</td>
</tr>
<tr>
<td>CA ([2H10]DPHM)</td>
<td>125.2 (3.8)</td>
<td>177.8 (20.4)</td>
<td>184.9 (7.9)</td>
<td>N/A</td>
<td>256.6 (5.0)</td>
</tr>
<tr>
<td>UV (DPHM)</td>
<td>31.5 (3.2)</td>
<td>N/A</td>
<td>N/A</td>
<td>137.9 (6.8)</td>
<td>N/A</td>
</tr>
<tr>
<td>UV ([2H10]DPHM)</td>
<td>47.3 (3.2)</td>
<td>N/A</td>
<td>N/A</td>
<td>180.9 (7.6)</td>
<td>N/A</td>
</tr>
<tr>
<td>MA (DPHM)</td>
<td>262.1 (9.9)</td>
<td>223.1 (9.5)</td>
<td>232.6 (8.9)</td>
<td>241.5 (8.2)</td>
<td>330.0 (6.6)</td>
</tr>
<tr>
<td>MA ([2H10]DPHM)</td>
<td>40.6 (2.1)</td>
<td>30.5 (5.0)</td>
<td>42.8 (1.7)</td>
<td>38.8 (1.5)</td>
<td>65.5 (1.8)</td>
</tr>
</tbody>
</table>

N/A - reference site could not be sampled due to catheter failure
Table 17: Pharmacokinetic parameters calculated during paired maternal/fetal infusions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>E#122Z</th>
<th>E#2101</th>
<th>E#2177</th>
<th>E#2181</th>
<th>E#2241</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUCmm (ng*min/mL)</td>
<td>95397</td>
<td>87278</td>
<td>88534</td>
<td>94710</td>
<td>128464</td>
<td>-</td>
</tr>
<tr>
<td>AUCff (ng*min/mL)</td>
<td>50872</td>
<td>87989</td>
<td>83072</td>
<td>149359</td>
<td>109036</td>
<td>-</td>
</tr>
<tr>
<td>AUCfm (ng*min/mL)</td>
<td>14089</td>
<td>12645</td>
<td>17516</td>
<td>15987</td>
<td>26466</td>
<td>-</td>
</tr>
<tr>
<td>AUCmf (ng*min/mL)</td>
<td>10888</td>
<td>7684</td>
<td>16805</td>
<td>45697</td>
<td>14847</td>
<td>-</td>
</tr>
<tr>
<td>MDRT (M) (min)</td>
<td>51.0</td>
<td>58.5</td>
<td>52.1</td>
<td>56.9</td>
<td>40.7</td>
<td>51.8 ± 3.1</td>
</tr>
<tr>
<td>MDRT (F) (min)</td>
<td>70.7</td>
<td>44.9</td>
<td>96.8</td>
<td>74.1</td>
<td>69.3</td>
<td>71.2 ± 8.2</td>
</tr>
<tr>
<td>α (M) (min⁻¹)</td>
<td>2.0</td>
<td>0.4</td>
<td>0.6</td>
<td>0.2</td>
<td>0.1</td>
<td>0.66 ± 0.35</td>
</tr>
<tr>
<td>T₁/₂ (min)</td>
<td>0.4</td>
<td>1.7</td>
<td>1.2</td>
<td>4.3</td>
<td>5.3</td>
<td>2.6 ± 0.9</td>
</tr>
<tr>
<td>α (F) (min⁻¹)</td>
<td>0.3</td>
<td>0.4</td>
<td>11.2</td>
<td>0.54</td>
<td>0.43</td>
<td>2.56 ± 2.14</td>
</tr>
<tr>
<td>T₁/₂ (min)</td>
<td>2.8</td>
<td>1.7</td>
<td>0.10</td>
<td>1.3</td>
<td>1.6</td>
<td>1.8 ± 0.3</td>
</tr>
<tr>
<td>β (M) (min⁻¹)</td>
<td>0.009</td>
<td>0.009</td>
<td>0.008</td>
<td>0.010</td>
<td>0.016</td>
<td>0.010 ± 0.001</td>
</tr>
<tr>
<td>T₁/₂ (min)</td>
<td>77.0</td>
<td>77.0</td>
<td>86.6</td>
<td>69.3</td>
<td>43.3</td>
<td>77.5 ± 3.2</td>
</tr>
<tr>
<td>β (F) (min⁻¹)</td>
<td>0.010</td>
<td>0.016</td>
<td>0.010</td>
<td>0.016</td>
<td>0.019</td>
<td>0.014 ± 0.002</td>
</tr>
<tr>
<td>T₁/₂ (min)</td>
<td>64.3</td>
<td>43.3</td>
<td>69.3</td>
<td>43.3</td>
<td>36.5</td>
<td>52.3 ± 7.0</td>
</tr>
<tr>
<td>CLT (M) (mL/min)</td>
<td>2750</td>
<td>3000</td>
<td>2960</td>
<td>2770</td>
<td>2040</td>
<td>2704 ± 173</td>
</tr>
<tr>
<td>CLT (F) (mL/min)</td>
<td>1300</td>
<td>750</td>
<td>790</td>
<td>440</td>
<td>610</td>
<td>778 ± 144</td>
</tr>
<tr>
<td>CLT (M) (mL/min/Kg)</td>
<td>43.9</td>
<td>36.7</td>
<td>39.5</td>
<td>42.1</td>
<td>30.2</td>
<td>38.5 ± 2.4</td>
</tr>
<tr>
<td>CLT (F) (mL/min/Kg)</td>
<td>575.2</td>
<td>364.8</td>
<td>352.0</td>
<td>202.2</td>
<td>250.0</td>
<td>348.9 ± 64.2</td>
</tr>
</tbody>
</table>

* The value of α calculated in E2177 was taken as an outlier and the value recalculated excluding this animal.
Table 18: The uncorrected and corrected (i.e., for drug lost via the placenta) volumes of distribution of DPHM in maternal sheep and $[^2\text{H}_{10}]$DPHM in fetal sheep following a simultaneous fetal infusion of $[^2\text{H}_{10}]$DPHM (170 μg/min) and maternal infusion of DPHM (670 μg/min).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>E#122Z</th>
<th>E#2101</th>
<th>E#2177</th>
<th>E#2181</th>
<th>E#2241</th>
<th>Mean ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maternal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vdss (L)</td>
<td>140.2</td>
<td>185.8</td>
<td>158.6</td>
<td>167.8</td>
<td>86.5</td>
<td>147.8 ± 17.0</td>
</tr>
<tr>
<td>Vdss (L/Kg)</td>
<td>2.2</td>
<td>2.3</td>
<td>1.6</td>
<td>2.6</td>
<td>1.3</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>Vdss' (L)</td>
<td>132.0</td>
<td>182.6</td>
<td>151.6</td>
<td>154.7</td>
<td>83.8</td>
<td>140.9 ± 16.4</td>
</tr>
<tr>
<td>Vdss' (L/Kg)</td>
<td>2.1</td>
<td>2.3</td>
<td>1.6</td>
<td>2.4</td>
<td>1.2</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td><strong>Fetal</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vdss (L)</td>
<td>91.7</td>
<td>34.2</td>
<td>95.8</td>
<td>36.3</td>
<td>46.1</td>
<td>60.8 ± 13.6</td>
</tr>
<tr>
<td>Vdss (L/Kg)</td>
<td>40.6</td>
<td>16.6</td>
<td>42.7</td>
<td>16.7</td>
<td>18.9</td>
<td>27.1 ± 5.9</td>
</tr>
<tr>
<td>Vdss' (L)</td>
<td>37.5</td>
<td>18.2</td>
<td>28.0</td>
<td>13.8</td>
<td>11.7</td>
<td>21.8 ± 4.8</td>
</tr>
<tr>
<td>Vdss' (L/Kg)</td>
<td>13.6</td>
<td>8.8</td>
<td>12.5</td>
<td>6.3</td>
<td>4.8</td>
<td>9.2 ± 1.7</td>
</tr>
</tbody>
</table>

Vdss' Volume of distribution at steady-state corrected for fraction of maternal dose lost to the fetus and the fraction of the fetal dose lost to the mother.
Figure 39: Femoral arterial plasma concentrations of DPHM and [\(^{2}\text{H}_{10}\)]DPHM in fetus and mother following simultaneous infusion of DPHM (670 \(\mu\)g/min) via the maternal femoral vein and [\(^{2}\text{H}_{10}\)]DPHM (170 \(\mu\)g/min) via the fetal lateral tarsal vein.
Figure 40: A representative plot of the concentrations of DPHM and $[^2\text{H}10]$DPHM in amniotic fluid and fetal tracheal fluid following simultaneous infusion of DPHM (670 $\mu$g/min) via the maternal femoral vein and $[^2\text{H}10]$DPHM (170 $\mu$g/min) via the fetal lateral tarsal vein (E#2181). AMN - amniotic fluid and TR - fetal tracheal fluid.
Table 19: Weight corrected trans- and non-placental clearances of DPHM in fetus and mother following a simultaneous infusion of DPHM to mother and $[^{2}H_{10}]$DPHM to the fetus (mL/min/Kg).

<table>
<thead>
<tr>
<th>EWE</th>
<th>CLmm (mL/min/Kg)</th>
<th>CLff (mL/min/Kg)</th>
<th>CLmf (mL/min/Kg)</th>
<th>CLfm (mL/min/Kg)</th>
<th>CLmo (mL/min/Kg)</th>
<th>CLfo (mL/min/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E#2177</td>
<td>40.7</td>
<td>441.1</td>
<td>60.1</td>
<td>308.8</td>
<td>38.9</td>
<td>129.2</td>
</tr>
<tr>
<td>E#2101</td>
<td>38.9</td>
<td>370.6</td>
<td>26.9</td>
<td>173.4</td>
<td>38.2</td>
<td>197.3</td>
</tr>
<tr>
<td>E#122z</td>
<td>43.4</td>
<td>471.9</td>
<td>47.6</td>
<td>280.2</td>
<td>41.3</td>
<td>191.7</td>
</tr>
<tr>
<td>E#2181</td>
<td>44.8</td>
<td>225.0</td>
<td>105.6</td>
<td>139.3</td>
<td>41.3</td>
<td>85.7</td>
</tr>
<tr>
<td>E#2241</td>
<td>31.5</td>
<td>272.6</td>
<td>28.2</td>
<td>203.5</td>
<td>30.4</td>
<td>69.1</td>
</tr>
<tr>
<td>Mean</td>
<td>39.9</td>
<td>356.2</td>
<td>53.7</td>
<td>221.0</td>
<td>38.0</td>
<td>134.6</td>
</tr>
<tr>
<td>SEM</td>
<td>2.3</td>
<td>47.4</td>
<td>14.4</td>
<td>32.0</td>
<td>2.0</td>
<td>26.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>EWE</th>
<th>CA (mL/min/Kg)</th>
<th>CA (mL/min/Kg)</th>
<th>CA (mL/min/Kg)</th>
<th>CA (mL/min/Kg)</th>
<th>CA (mL/min/Kg)</th>
<th>CA (mL/min/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E#2177</td>
<td>40.9</td>
<td>450.4</td>
<td>67.5</td>
<td>315.7</td>
<td>38.8</td>
<td>127.1</td>
</tr>
<tr>
<td>E#2101</td>
<td>35.8</td>
<td>427.4</td>
<td>31.6</td>
<td>253.5</td>
<td>35.0</td>
<td>173.9</td>
</tr>
<tr>
<td>E#122z</td>
<td>43.4</td>
<td>513.8</td>
<td>48.2</td>
<td>305.5</td>
<td>41.3</td>
<td>208.3</td>
</tr>
<tr>
<td>E#2181</td>
<td>31.3</td>
<td>292.1</td>
<td>27.5</td>
<td>220.7</td>
<td>30.3</td>
<td>71.5</td>
</tr>
<tr>
<td>Mean</td>
<td>37.8</td>
<td>420.9</td>
<td>43.7</td>
<td>273.8</td>
<td>36.4</td>
<td>145.2</td>
</tr>
<tr>
<td>SEM</td>
<td>2.7</td>
<td>46.7</td>
<td>9.1</td>
<td>22.3</td>
<td>2.4</td>
<td>29.7</td>
</tr>
</tbody>
</table>

Note: Carotid arterial samples were not available in ewe 2181.
Weight corrected clearances (mL/min/Kg) are normalized to maternal (CLmm and CLmo) or fetal (CLff, CLfm, CLmf, and CLfo) body weight estimated at the time of experimentation.
Note: The CLmm is not equal to CLmo + CLmf because CLmf is weight corrected to fetal weight rather than maternal weight.
Table 20: Non weight corrected and weight corrected trans- and non-placental clearance parameters of DPHM following simultaneous infusions of DPHM and \([^{2}\text{H}_{10}]\text{DPHM}\) to mother and fetus, respectively. Calculated using the mass balance approach.

<table>
<thead>
<tr>
<th>EWE</th>
<th>CLmf (mL/min/Kg)</th>
<th>CLfm (mL/min/Kg)</th>
<th>CLmo (mL/min/Kg)</th>
<th>CLfo (mL/min/Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E#122z</td>
<td>69</td>
<td>344</td>
<td>44</td>
<td>257</td>
</tr>
<tr>
<td>E#2101</td>
<td>34</td>
<td>220</td>
<td>37</td>
<td>154</td>
</tr>
<tr>
<td>E#2177</td>
<td>70</td>
<td>291</td>
<td>39</td>
<td>83</td>
</tr>
<tr>
<td>E#2181</td>
<td>105</td>
<td>144</td>
<td>41</td>
<td>72</td>
</tr>
<tr>
<td>E#2241</td>
<td>29</td>
<td>212</td>
<td>30</td>
<td>47</td>
</tr>
<tr>
<td>Mean</td>
<td>62</td>
<td>242</td>
<td>38.3</td>
<td>123</td>
</tr>
<tr>
<td>SEM</td>
<td>14</td>
<td>34</td>
<td>2.4</td>
<td>38</td>
</tr>
</tbody>
</table>

Weight corrected clearances (mL/min/Kg) are normalized to maternal (CLmm and CLmo) or fetal (CLff, CLfm, CLmf, and CLfo) body weight.

In the two animals which had umbilical arterial transit time flow probes and functional umbilical venous catheters (ewes 122z and 2181), trans-placental clearances were calculated using the Fick method. The extraction ratios of \([^{2}\text{H}_{10}]\text{DPHM}\) and DPHM were 0.64 ± 0.02 and -0.16 ± 0.05 in ewe 122Z, and 0.51 ± 0.01 and -0.23 ± 0.03 in ewe 2181, respectively. The corresponding values for CLmf were 31.6 ± 9.5 [ewe 122z] and 68.7 ± 7.4 [ewe 2181] mL/min/Kg, and for CLfm were 126.2 ± 4.4 [ewe 122z] and 153 ± 3.9 [ewe 2181] mL/min/Kg.

The values for CLmf calculated using the Fick method are lower in both animals (35 and 33% in ewes 122z and 2181, respectively) compared to values calculated using the compartmental method. The Fick estimate for CLfm was equivalent to the model estimate in ewe 2181, but underestimated the model estimate by a factor of two in ewe 122z (Table 21). The ratio of fetal to maternal placental clearances (i.e., CLfm/CLmf) from the Fick clearance estimates were 4.0
and 2.2 vs. the 2 compartment-open model estimates, which were 6.0 and 1.3 in ewes 122z and 2181, respectively.

Table 21: A comparison of model dependent estimates of trans-placental clearances (2 compartment-open model), and model independent (Fick method) estimates of trans-placental clearances.

<table>
<thead>
<tr>
<th></th>
<th>E#122Z</th>
<th>E#2181</th>
<th>Yoo et al. (E#130)</th>
<th>Mean (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extraction (M to F)</td>
<td>-0.16</td>
<td>-0.23</td>
<td>-0.33</td>
<td>-0.24 ± 0.05</td>
</tr>
<tr>
<td>Extraction (F to M)</td>
<td>0.64</td>
<td>0.51</td>
<td>0.65</td>
<td>0.60 ± 0.05</td>
</tr>
<tr>
<td>Umbilical Arterial Flow (mL/min/Kg)</td>
<td>195.4</td>
<td>294.9</td>
<td>a</td>
<td>b280.7 ± 39.1</td>
</tr>
<tr>
<td>CLmf (Fick)</td>
<td>31.6</td>
<td>68.7</td>
<td>c92.6</td>
<td>64.3 ± 17.7</td>
</tr>
<tr>
<td>CLfm (Fick)</td>
<td>126.2</td>
<td>153.0</td>
<td>c182.4</td>
<td>153.9 ± 16.2</td>
</tr>
<tr>
<td>CLmf (model)</td>
<td>47.6</td>
<td>105.6</td>
<td>d27.5</td>
<td>60.2 ± 23.4</td>
</tr>
<tr>
<td>CLfm (model)</td>
<td>280.2</td>
<td>139.2</td>
<td>d133.8</td>
<td>184.4 ± 47.9</td>
</tr>
</tbody>
</table>

a - umbilical arterial blood flow was not measured in this experiment.
b - mean blood flow includes data for an animal in which the umbilical venous catheter was not functioning, thus, extraction could not be calculated for this animal.
c - Fick clearance estimates are calculated using the extraction ratio from the previous experiment and the mean umbilical arterial blood flow from the current experiment.
d - model estimates obtained from an earlier experiment (Yoo et al., 1989)

The clearance parameters were also calculated in four animals using the carotid arterial plasma drug concentrations rather than femoral arterial drug concentrations. Although there appeared to be an overall increase in the mean values for CLff, CLfm, and CLfo using carotid arterial plasma concentrations compared to the clearance parameters calculated using the femoral arterial reference plasma drug concentrations, these differences were not statistically significant (p>0.05 Paired sample T-test) (Table 19).
3.2.4.2. Metabolism of DPHM to DPMA in Mother and Fetus

A mean (± SEM) arterial plasma concentration vs. time plots of DPMA and [\(^2\text{H}_{10}\)DPMA in maternal and fetal femoral arterial plasma samples are shown in figure 41 and 42. Although concentrations of DPHM and [\(^2\text{H}_{10}\)DPHM reached steady-state at 150 minutes from the start of the infusion, the concentrations of DPMA and [\(^2\text{H}_{10}\)DPMA did not reach steady-state at this time, and continued to increase for 30-120 minutes following the end of the infusion. At all time points during the infusion, the concentration of [\(^2\text{H}_{10}\)DPMA was higher in the fetus than in the mother, whereas the opposite was true in maternal plasma (i.e., DPMA was higher than [\(^2\text{H}_{10}\)DPMA in maternal plasma). The peak concentration of DPMA in maternal and fetal plasma averaged 137.4 ± 18.5 and 92.8 ± 16.8 ng/mL, respectively, while the peak maternal and fetal plasma levels of [\(^2\text{H}_{10}\)DPMA were 28.7 ± 4.3 and 135.0 ± 20.1 ng/mL (statistically significant difference; Paired Sample T-test p<0.05), respectively. The time at which the peak levels occurred post-infusion was 18.0 ± 7.3 minutes for both labeled and unlabeled DPMA in the ewe, whereas in the fetus the value was 87.0 ± 14.5 minutes. Following the peak, the metabolite levels in the fetus declined much more slowly than in the ewe (Figure 41). The model estimates for DPHM, [\(^2\text{H}_{10}\)DPHM, DPMA, and [\(^2\text{H}_{10}\)DPMA following simultaneous fitting of the parent drug and metabolite data are shown in table 22. The apparent elimination half-lives of the metabolite were 180.3 ± 13.0 min (3.0 ± 0.2 hrs) and 911.4 ± 151.5 min (15.2 ± 2.5 hrs) in mother and fetus, respectively. These values were significantly different (p<0.05, Paired Sample T-test). The formation rate constant (Kf) could not be calculated because the volume of distribution of the metabolite was not known. The AUC ratio of DPMA to DPHM was significantly less than the AUC ratio of [\(^2\text{H}_{10}\)DPMA/[\(^2\text{H}_{10}\)DPHM in maternal plasma (i.e., 0.62
146

± 0.07 vs. 0.93 ± 0.12) [Paired Sample T-test, p<0.05]. Although amniotic and fetal lung fluid samples were analyzed for the presence of the metabolite, it was never detected. The extraction of DPMA and [²H₁₀]DPMA across the placenta was -0.07 ± 0.06 and -0.02 ± 0.01 for ewe 122z and -0.07 ± 0.01 and -0.09 ± 0.01 for ewe 2181, respectively.

Table 22: Pharmacokinetic parameters calculated from the simultaneous fitting of DPHM and DPMA in maternal plasma and [²H₁₀]DPHM and [²H₁₀]DPMA in fetal plasma. (n=5).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>E#2241</th>
<th>E#2177</th>
<th>E#2181</th>
<th>E#2101</th>
<th>E#122z</th>
<th>Mean</th>
<th>SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>maternal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K₁₂(min⁻¹)</td>
<td>0.0179</td>
<td>0.0619</td>
<td>0.1343</td>
<td>0.3128</td>
<td>0.324</td>
<td>0.170</td>
<td>0.064</td>
</tr>
<tr>
<td>K₂₁(min⁻¹)</td>
<td>0.0204</td>
<td>0.0146</td>
<td>0.0276</td>
<td>0.1648</td>
<td>0.0413</td>
<td>0.054</td>
<td>0.028</td>
</tr>
<tr>
<td>K₁₀(min⁻¹)</td>
<td>0.0603</td>
<td>0.1576</td>
<td>0.1312</td>
<td>0.7086</td>
<td>0.3192</td>
<td>0.275</td>
<td>0.117</td>
</tr>
<tr>
<td>Fm (min*L⁻¹)</td>
<td>0.0011</td>
<td>0.0005</td>
<td>0.0011</td>
<td>0.0014</td>
<td>0.0007</td>
<td>0.001</td>
<td>0.0002</td>
</tr>
<tr>
<td>Vc (L)</td>
<td>33.6</td>
<td>18.4</td>
<td>20.9</td>
<td>4.3</td>
<td>9.5</td>
<td>17.3</td>
<td>5.1</td>
</tr>
<tr>
<td>Km(min⁻¹)</td>
<td>0.0037</td>
<td>0.0031</td>
<td>0.0038</td>
<td>0.0047</td>
<td>0.0043</td>
<td>0.0039</td>
<td>0.0003</td>
</tr>
<tr>
<td>T½ (min)</td>
<td>187.3</td>
<td>223.5</td>
<td>182.4</td>
<td>147.4</td>
<td>161.1</td>
<td>180.4</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td>fetal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K₁₂(min⁻¹)</td>
<td>0.3814</td>
<td>0.1092</td>
<td>0.7442</td>
<td>0.8033</td>
<td>1.067</td>
<td>0.621</td>
<td>0.169</td>
</tr>
<tr>
<td>K₂₁(min⁻¹)</td>
<td>0.0172</td>
<td>0.0221</td>
<td>0.0426</td>
<td>0.1603</td>
<td>0.0681</td>
<td>0.062</td>
<td>0.026</td>
</tr>
<tr>
<td>K₁₀(min⁻¹)</td>
<td>0.4290</td>
<td>0.0877</td>
<td>0.3211</td>
<td>0.1705</td>
<td>0.3196</td>
<td>0.266</td>
<td>0.061</td>
</tr>
<tr>
<td>Fm (min*L⁻¹)</td>
<td>0.0012</td>
<td>0.0028</td>
<td>0.0029</td>
<td>0.0025</td>
<td>0.0027</td>
<td>0.0024</td>
<td>0.0003</td>
</tr>
<tr>
<td>Vc (L)</td>
<td>1.4</td>
<td>9.8</td>
<td>1.4</td>
<td>4.5</td>
<td>3.8</td>
<td>4.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Km(min⁻¹)</td>
<td>0.0011</td>
<td>0.0006</td>
<td>0.0005</td>
<td>0.0010</td>
<td>0.0010</td>
<td>0.0008</td>
<td>0.0001</td>
</tr>
<tr>
<td>T½ (min)</td>
<td>630.0</td>
<td>1155.0</td>
<td>1386.0</td>
<td>693.0</td>
<td>693.0</td>
<td>911.4</td>
<td>151.9</td>
</tr>
</tbody>
</table>
Figure 41: Mean (± SEM) maternal and fetal femoral arterial plasma concentrations of DPMA and [²H₁₀]DPMA following simultaneous fetal and maternal infusions of [²H₁₀]DPHM (170 μg/min) and DPHM (670 μg/min), respectively.
Figure 42: Maternal and fetal femoral arterial plasma concentrations of DPMA and $[^{2}\text{H}_{10}]$DPMA following simultaneous fetal and maternal infusions of $[^{2}\text{H}_{10}]$DPHM and DPHM respectively (x-axis scaling is reduced to show initial formation of metabolite).
3.2.4.3. Renal Clearance of DPHM, [²H₁₀]DPHM, DPMA and [²H₁₀]DPMA

The fetal and maternal urine flow data collected during and following the infusion are shown in table 23, while the renal clearances calculated for DPHM, [²H₁₀]DPHM, DPMA, and [²H₁₀]DPMA in both ewe and fetus are given in table 24. The cumulative excretion plot for DPHM and [²H₁₀]DPHM in maternal and fetal urine clearly shows a plateau following the infusion (Figure 43). The weight corrected renal clearance of DPHM was \(~200\) fold less in maternal sheep compared to fetal lambs (Table 24). The contribution of DPHM clearance to the maternal non-placental clearance was \(0.02 \pm 0.01\%\), while in the fetus the contribution was \(2.3 \pm 0.4\%\) (Table 24). The metabolites, DPMA and [²H₁₀]DPMA, were both present in adult urine during and following the infusion. In marked contrast to DPHM and [²H₁₀]DPHM, these metabolites (DPMA and [²H₁₀]DPMA) were present in only small quantities in fetal urine and often were not detectable. The cumulative amount of DPMA and [²H₁₀]DPMA excreted in fetal urine appears to increase following the cessation of fetal urine collection; therefore, \(\Sigma M_u\) is likely somewhat underestimated in the fetal lamb. In addition, the excretion of the metabolite continues to increase in the ewe for some time following the infusion, and only appears to be near the plateau of the cumulative urinary excretion plot at the end of the experimental protocol (Figure 44). Thus, it appears that the renal clearance of DPMA is not entirely complete during the end of the experimental protocol. While an estimation of the renal clearance of DPMA in both mother and fetus may have been underestimated, the weight corrected relative renal clearance of the DPMA was \(~50\) fold greater in mother when compared to the fetus. The percentage of the dose excreted as DPMA was calculated based on the ratio of the dose administered and the cumulative amount of DPMA (corrected for mass difference between parent drug and metabolite) excreted in the urine at time infinity (\(i.e.,\) fraction of dose = Dose DPHM/(\(\Sigma M_u\) DPMA*Mass Correction Factor). Although this estimate may underestimate the contribution of the renal clearance of DPMA due to an underestimation of \(\Sigma M_u\) DPMA, a
rough estimate suggests that around 1% of maternal dose can be accounted for by the renal elimination of DPMA.
Table 23: Fetal and maternal urine flow during paired fetal and maternal infusions of DPHM and \([^{2}H_{10}]DPHM\), respectively.

<table>
<thead>
<tr>
<th></th>
<th>Maternal</th>
<th>Fetal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Urine flow (mL/hr)</td>
<td>Urine flow (mL/hr/Kg)</td>
</tr>
<tr>
<td>E#122Z</td>
<td>75.7 ± 13.0</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>E#2101</td>
<td>84.5 ± 11.7</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>E#2177</td>
<td>44.3 ± 3.8</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>E#2181</td>
<td>57.6 ± 3.1</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>E#2241</td>
<td>35.4 ± 3.3</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>59.5 ± 9.3</td>
<td>0.8 ± 0.1</td>
</tr>
</tbody>
</table>

Values include the urine flow during the infusion and the post infusion sampling period.
Table 24: The renal clearances of DPHM and DPMA, and $[^2\text{H}_{10}]$DPHM and $[^2\text{H}_{10}]$DPMA in adult sheep and in fetal lambs.

<table>
<thead>
<tr>
<th></th>
<th>DPHM</th>
<th>$[^2\text{H}_{10}]$DPHM</th>
<th>$[^2\text{H}_{10}]$DPHM</th>
<th>DPMA</th>
<th>$[^2\text{H}_{10}]$DPMA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(maternal)</td>
<td>(maternal)</td>
<td>% CLmo</td>
<td>(fetal)</td>
<td>(fetal)</td>
</tr>
<tr>
<td></td>
<td>mL/min/Kg</td>
<td>mL/min/Kg</td>
<td></td>
<td>mL/min/Kg</td>
<td>mL/min/Kg</td>
</tr>
<tr>
<td>Ewe</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>nd</td>
<td>nd</td>
<td>0</td>
<td>1.43</td>
<td>1.80</td>
</tr>
<tr>
<td></td>
<td>0.009</td>
<td>nd</td>
<td>0.023</td>
<td>4.09</td>
<td>3.64</td>
</tr>
<tr>
<td></td>
<td>0.023</td>
<td>nd</td>
<td>0.053</td>
<td>2.81</td>
<td>1.96</td>
</tr>
<tr>
<td></td>
<td>0.014</td>
<td>nd</td>
<td>0.031</td>
<td>1.31</td>
<td>1.85</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>nd</td>
<td>0.003</td>
<td>2.70</td>
<td>2.21</td>
</tr>
<tr>
<td>Mean</td>
<td>0.012</td>
<td></td>
<td>0.020</td>
<td>2.47</td>
<td>2.29</td>
</tr>
<tr>
<td>SEM</td>
<td>0.005</td>
<td></td>
<td>0.009</td>
<td>0.51</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>DPHM</td>
<td>$[^2\text{H}_{10}]$DPHM</td>
<td>$[^2\text{H}_{10}]$DPHM</td>
<td>DPMA</td>
<td>$[^2\text{H}_{10}]$DPMA</td>
</tr>
<tr>
<td></td>
<td>(maternal)</td>
<td>(maternal)</td>
<td>% Dose Excreted</td>
<td>(fetal)</td>
<td>(fetal)</td>
</tr>
<tr>
<td></td>
<td>mL/min/Kg</td>
<td>mL/min/Kg</td>
<td></td>
<td>mL/min/Kg</td>
<td>mL/min/Kg</td>
</tr>
<tr>
<td>E#2101</td>
<td>1.98</td>
<td>2.02</td>
<td>1.01</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>E#122Z</td>
<td>1.00</td>
<td>1.08</td>
<td>0.97</td>
<td>0.008</td>
<td>nd</td>
</tr>
<tr>
<td>E#2177</td>
<td>0.52</td>
<td>0.51</td>
<td>0.69</td>
<td>0.056</td>
<td>nd</td>
</tr>
<tr>
<td>E#2181</td>
<td>0.54</td>
<td>0.57</td>
<td>1.11</td>
<td>0.003</td>
<td>nd</td>
</tr>
<tr>
<td>E#2241</td>
<td>0.42</td>
<td>0.63</td>
<td>0.95</td>
<td>0.005</td>
<td>nd</td>
</tr>
<tr>
<td>Mean</td>
<td>0.89</td>
<td>0.96</td>
<td>0.95</td>
<td>0.0181</td>
<td>nd</td>
</tr>
<tr>
<td>SEM</td>
<td>0.29</td>
<td>0.28</td>
<td>0.07</td>
<td>0.0128</td>
<td>0.011</td>
</tr>
</tbody>
</table>

nd - intact drug or metabolite was below the minimal quantifiable concentration of the analysis method.

The % contribution to CLfo and CLmo for intact drug is based on the calculated weight corrected renal clearance of intact drug (DPHM in mother and $[^2\text{H}_{10}]$DPHM in fetus) and the weight corrected maternal and fetal non-placental clearances calculated using the method of Szeto (1982).

The % contribution of metabolite excretion to fetal and maternal dose is based on the weight corrected total amount of metabolite excreted in fetal and maternal urine, and the dose of $[^2\text{H}_{10}]$DPHM and DPHM administered to mother and fetus, respectively.
Figure 43: Mean (± SEM) cumulative amounts of DPHM in maternal urine and $[^{2}H_{10}]$DPHM in fetal urine following simultaneous fetal infusion of $[^{2}H_{10}]$DPHM (170 µg/min) and maternal infusions of DPHM (670 µg/min) (n=5). [total fetal dose = 66.2 mg and total maternal dose = 261.2 mg].
Figure 44: Mean (± SEM) cumulative amounts of DPMA in maternal urine and [²H₁₀]DPMA in fetal urine following simultaneous fetal and maternal infusions of [²H₁₀]DPHM (170 μg/min) and DPHM (670 μg/min) respectively (n=5). [total fetal dose = 66.2 mg and total maternal dose = 261.2 mg].
3.2.4.4. Fetal Effects Following Fetal and Maternal Paired Infusions of $[^2\text{H}]\text{DPHM and DPHM}$, Respectively.

There were no consistent changes from the controls (i.e., 6 hours pre infusion) in fetal arterial pressure (52.4 ± 1.7 mm Hg), umbilical blood flow (280.7 ± 39.1 mL/min/Kg; n=3), fetal urine flow (0.45 ± 0.10 mL/min), and maternal and fetal urine pH (7.62 ± 0.07 and 6.78 ± 0.10, respectively) during the experiment. However, there was a reduction in fetal heart rate during the course of the infusion. The fetal heart rate increased following the end of the infusion (repeated measures ANOVA p<0.05) (Figure 46). In addition, a statistically significant increase in intermediate electrocortical activity was noted, with a corresponding reduction in high and low voltage states; however, decreases in high and low ECoG activity did not reach statistical significance (Figure 45). There was also a trend for the reduction of fetal breathing movements, but these differences did not reach statistical significance (Figure 46).
Figure 45: Mean (± SEM) high, intermediate, and low electrocortical activity in fetal sheep following simultaneous fetal and maternal infusions of $[^2H_{10}]$DPHM and DPHM, respectively. (n=4)
Figure 46: Mean (± SEM) fetal heart rate and fetal breathing movements following simultaneous fetal and maternal infusions of [2H10]DPHM and DPHM, respectively. (n=5)
3.3. *In Vitro* Studies

3.3.1. Uptake of DPHM in Blood Cells

The ratio of DPHM plasma concentration to DPHM BC concentration as a function of time is shown in figure 47. The time required to reach equilibrium was rapid (*i.e.*, <2.5 minutes). The ratio at equilibrium was close to 1, suggesting that the plasma concentration is a good reflection of the whole blood concentration of DPHM. The experiment investigating the effect of temperature on DPHM uptake into BCs was to ensure that sample handling techniques (*i.e.*, allowing the collected samples to cool to room temperature prior to separating the BC and plasma fractions) did not change the plasma DPHM concentration due to BC uptake. The ratio of DPHM in plasma to DPHM in BC was not significantly altered during when measured at either 25 or 39 °C.

3.3.2. Plasma Protein Binding of DPMA

An equilibration time of 8 hours was required for the determination of the plasma protein binding of DPMA. No significant volume shifts were associated with this equilibrium time, while volume shifts did occur for longer dialysis times (*i.e.*, 24 and 36 hours). Non-specific binding of DPMA to the equilibrium dialysis cell and the membrane could not be detected. The metabolite was highly bound in both maternal and fetal plasma, with the percent bound being 99.4 ± 0.01% and 98.9 ± 0.07% for the five replicates, respectively (n=5). The free fraction of DPMA in adult plasma was 0.006 ± 0.002, while the free fraction of DPMA in fetal plasma was 0.010 ± 0.001.
3.3.3. Fetal and Adult Hepatic Microsomal Metabolism of DPHM

The metabolism of DPHM was examined using hepatic microsomes prepared from non-pregnant adult sheep and fetal lambs. The formation of N-demethyl DPHM was monitored using a previously published analytical method (Abernethy and Greenblatt, 1983, and Blyden et al, 1986). This method was adapted for GC-MS application from GC-NPD. The fragment ions $m/z$ 165 and 167 were monitored for N-demethyl DPHM. Quantitation of N-demethyl DPHM over a concentration range of 2.5 to 250.0 ng/mL was not possible due to the non-linear nature of the calibration curve above concentrations of 50.0 ng/mL. Thus, measurements of N-demethyl DPHM were made within the linear range of the calibration curve (i.e., 2.5 to 50.0 ng/mL).

The microsomal protein concentration and P450 content are presented in table 25. The gestational ages of the fetuses used in this experiment were between 131 and 138 days (term = 145
days). Although both protein concentrations and P450 concentrations could be measured in adult microsomes, only the protein concentration could be measured in the fetal microsomes. This was due to both the contamination of the fetal microsomal preparation with hemoglobin and the low amount of P450 present in the fetal microsomes.

All incubations were carried out at 39°C. The incubation protocol was optimized for protein concentration, substrate concentration, and time. Incubations were optimized for 2.0 nmoles of P450. Because it was not possible to accurately estimate the P450 content in fetal microsomes, incubations were carried out with 1.0 mg of microsomal protein from either fetal or adult source. The amount of substrate used was 0.5 μmoles. No product (i.e., N-demethyl DPHM) was formed in incubations containing boiled microsomes, microsomal protein with no co-factors (i.e., NADH and NADPH), or no microsomal protein. Fetal and adult time course experiments are shown in figures 48 and 49. In adult microsomes, the amount of DPHM was found to decrease; however, the quantity of DPHM which was consumed did not correlate well with the N-demethyl DPHM formed; that is, only 10% of the missing DPHM could be accounted for by N-demethyl DPHM. In addition, N,N-didemethyl DPHM was not detected. Fetal microsomal incubations resulted in less of a reduction in the amount of DPHM over time, and smaller amounts of N-demethyl DPHM were formed. The concentrations of N-demethyl DPHM formed could not be accurately estimated using the present chromatographic method due to the co-elution of the parent drug (very high concentration) with the metabolite (very low concentration). Subsequent incubations in both fetal and adult microsomes were conducted for 90 minutes with 0.1 μmoles of substrate. These studies also demonstrated that the formation of N-demethyl DPHM was roughly seven fold less (84% less) in hepatic microsomes prepared from fetal lambs compared to those from adult sheep (Figure 50). However, a similar amount of DPMA was produced in both fetal and adult microsomes (Figure 51).
Table 25: Protein and Cytochrome P450 concentrations in fetal and adult microsomes.

<table>
<thead>
<tr>
<th>Animal #</th>
<th>P450 content (nmol/mL)</th>
<th>Protein Conc. (mg/mL)</th>
<th>P450/protein (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult #1</td>
<td>22.8</td>
<td>13.4</td>
<td>2.13</td>
</tr>
<tr>
<td>Adult #2</td>
<td>28.5</td>
<td>11.4</td>
<td>2.50</td>
</tr>
<tr>
<td>Adult #3</td>
<td>32.6</td>
<td>10.4</td>
<td>3.13</td>
</tr>
<tr>
<td>Adult #4</td>
<td>15.5</td>
<td>10.1</td>
<td>1.53</td>
</tr>
<tr>
<td>Adult #5</td>
<td>24.4</td>
<td>13.2</td>
<td>1.85</td>
</tr>
<tr>
<td>Adult #6</td>
<td>14.8</td>
<td>8.5</td>
<td>1.74</td>
</tr>
<tr>
<td>Fetus #230</td>
<td>na</td>
<td>3.92</td>
<td>na</td>
</tr>
<tr>
<td>Fetus #562</td>
<td>na</td>
<td>5.02</td>
<td>na</td>
</tr>
<tr>
<td>Fetus #1143</td>
<td>na</td>
<td>5.21</td>
<td>na</td>
</tr>
</tbody>
</table>
Figure 48: Time course of DPHM disappearance and N-demethyl DPHM production in adult microsomes.

Figure 49: Time course of DPHM disappearance in fetal microsomes.
Figure 50: Production of N-demethyl DPHM in fetal and maternal microsomes following a 90 minute incubation.

Figure 51: Production of DPMA in fetal and maternal microsomes following a 90 minute incubation.
4. Discussion

4.1. Development of a Gas Chromatographic-Mass Spectrometric Method for the Simultaneous Quantitation of DPHM and \([^2\text{H}_{10}]\text{DPHM}\) from Biological Fluids Obtained from Pregnant Sheep

The simultaneous co-administration of stable isotope labeled and unlabeled drug offers several advantages over traditional experimental designs where only unlabeled drug is available, particularly in investigations of fetal/maternal pharmacokinetics (See Section 1.6.). In order to address many of the questions posed by previous studies examining DPHM disposition in the ovine maternal/fetal unit, it was necessary to obtain a stable isotope labeled form of the drug. A synthetic method for tritium labeled DPHM has been reported in the literature (Blackburn and Ober, 1967); however, there are no reports describing the synthesis of a stable isotopically labeled form of the drug. Stable isotope labeling can be accomplished through a variety of means, including deuterium-hydrogen exchange, chemical synthesis, derivatization, and biosynthesis (Roncucci et al., 1976). Initial attempts to utilize deuterium exchange in the presence of a catalyst, aluminum chloride, resulted in the formation of only very small quantities of \([^2\text{H}_1]\text{DPHM}\) and \([^2\text{H}_2]\text{DPHM}\). Therefore, this approach was abandoned. Chemical synthesis appeared to be a better alternative for the production of \([^2\text{H}_{10}]\text{DPHM}\), particularly due to the high isotopic purity of the inexpensive synthetic precursor (i.e., \([^2\text{H}_6]\text{benzene} > 99.7\%\) isotopic purity). The synthesis method reported in this thesis provided sufficient quantities of both chemically and isotopically pure \([^2\text{H}_{10}]\text{DPHM}\) for experimental and analytical use.

The simultaneous administration of DPHM and \([^2\text{H}_{10}]\text{DPHM}\) to chronically instrumented pregnant sheep requires that there be an analytical method capable of differentiating between unlabeled and labeled drug when present together in a sample. Since the GC-NPD method used
in previous studies could not distinguish between \(^{[2}^\text{H}_{10}]\text{DPHM}\) and DPHM (Yoo et al., 1986), a new method was required. The most readily available and powerful (sensitive and selective) detection technique for routine quantitation of SIL compounds and their unlabeled counterparts is mass spectrometry (Chasseaud and Hawkins, 1990). To quantitate DPHM and \(^{[2}^\text{H}_{10}]\text{DPHM}\) independently in the same sample using mass spectrometry, the appropriate fragment ions must be selectively monitored. The ions selectively monitored should differentiate between labeled and unlabeled drug, be present in sufficient quantity (abundance) to provide the required sensitivity, and be free of undue chromatographic interference from co-eluting endogenous components. Following GC-MS/EI, DPHM and \(^{[2}^\text{H}_{10}]\text{DPHM}\) fragment extensively (Figure 8), as reported previously for DPHM (Chang et al., 1974). This limits the choice of fragment ions for SIM which meet the criterion outlined above. To avoid the extensive fragmentation, preliminary attempts were made to quantitate both DPHM and \(^{[2}^\text{H}_{10}]\text{DPHM}\) using GC-MS with methane positive chemical ionization (PCI). Methane PCI also resulted in extensive fragmentation of the compounds, and a further reduction in sensitivity; thus, EI ionization was used. The fragmentation of DPHM and \(^{[2}^\text{H}_{10}]\text{DPHM}\) following GC-MS/EI results in a base fragment ion of \(m/z\) 58 with no apparent molecular ions. The previously reported GC-MS analysis methods for DPHM all use the base fragment ion (\(m/z\) 58) for SIM (Carruthers et al., 1978, Rohdewald and Milsmann, 1986, Maurer and Pfleger, 1988, and Walters-Thompson and Mason, 1992). Since the deuterium labels of \(^{[2}^\text{H}_{10}]\text{DPHM}\) reside on the aromatic rings, the SIM of fragment \(m/z\) 58 could not provide the necessary differentiation between DPHM and \(^{[2}^\text{H}_{10}]\text{DPHM}\) (Figure 8). The fragment ions which meet the criteria for SIM analysis of DPHM and \(^{[2}^\text{H}_{10}]\text{DPHM}\) outlined above were determined to be \(m/z\) 165 (DPHM and orphenadrine) and \(m/z\) 173 (\(^{[2}^\text{H}_{10}]\text{DPHM}\)).
The sensitivity of a quantitative method employing mass spectrometry can be enhanced by using SIM rather than scanning the entire mass range (as outlined above). In addition, the sensitivity can be further augmented by the appropriate tuning algorithm. Several tuning algorithms were tested, including the auto, mid-mass, and manual tune. The manual-tune option optimized the mass spectrometer performance over the narrow mass range of \( m/z \) 100 to 219, rather than \( m/z \) 50 to 502 (auto-tune). This modification increased the sensitivity of the analytical method by a factor of approximately 20 fold. The use of both SIM and the appropriate tuning algorithm has provided the necessary sensitivity for the analysis of DPHM and \([2H_{10}]\text{DPHM}\).

A complication encountered during assay development resulted from the co-elution of N-demethyl DPHM with DPHM on an Ultra-2 capillary column (i.e., liquid phase 5% phenylmethylsilicone). Previously, using GC-NPD, N-demethyl DPHM was not detected; however, due to a similar fragmentation pattern (\( m/z \) 165 was also prominent for N-demethyl DPHM), N-demethyl DPHM now interfered with the quantitation of intact drug (Figure 9). Consequently, a capillary column with 5% phenylmethyl: 7% cyanopropylsilicone liquid phase coating (DB-1701) was used. This column provided the necessary separation of DPHM and \([2H_{10}]\text{DPHM}\) and their respective N-demethylated metabolites. The chromatography of the compounds was enhanced on this column through thermal trapping of analytes at the head of the column by keeping the initial column temperature low (140°C), as outlined by Sandra (1989). This resulted in sharper, narrower, and more symmetrical chromatographic peaks.

The narrower and sharper peaks produced by the enhanced efficiency of capillary GC requires that an additional precaution be considered when conducting quantitative mass spectrometry using SIM. Sufficient samples must be collected (scans/peak) over the time
window in which the chromatographic peak elutes, to ensure the accuracy of the peak shape and area (Pettit, 1986, and Falkner, 1982). The dwell time, i.e., the time the mass spectrometer focuses on one of the selected ions, should be adjusted so that at least 10-20 samples can be collected across the chromatographic peak of interest (Pettit, 1986, and Falkner, 1980). The current method uses a dwell time of 50 msec (~50 scans/peak) to ensure adequate sampling of the peaks of interest.

Utilization of $[^2\text{H}_{10}]$DPHM can result in "isotope effects" (i.e., the differentiation between the labeled and unlabeled compound) in vivo, in vitro, and within the GC-MS (Van Langenhove, 1986). Isotope effects were evident within the GC-MS both with regard to the elution and the fragmentation of $[^2\text{H}_{10}]$DPHM. $[^2\text{H}_{10}]$DPHM was found to elute more rapidly than DPHM (Figure 14). In addition, the abundance of the fragment $m/z$ 165 (DPHM) was greater than the corresponding fragment $m/z$ 173 for $[^2\text{H}_{10}]$DPHM (Figure 8). The lower abundance of the $m/z$ 173 fragment of $[^2\text{H}_{10}]$DPHM may be due to the breaking of a carbon-deuterium bond involved in the formation of this fragment. This deuterium-carbon bond is more stable than the equivalent carbon-hydrogen bond, and thus, render the molecule less likely to fragment in this fashion (Van Langenhove, 1986, and McCloskey et al., 1967). Because the fragment abundances for the corresponding ions (i.e., $m/z$ 165 and 173) were not equivalent, it was necessary to simultaneously evaluate standard calibration curves for both DPHM and $[^2\text{H}_{10}]$DPHM.

A common problem encountered during liquid-liquid extraction of tertiary amine analytes is non-specific binding to glassware (Smith and Stewart, 1981, and Jack, 1990). Numerous methods have been employed to prevent this phenomenon, including the use of silanized glassware. However, silanization is not always effective (Jack, 1980). The use of triethylamine
(TEA) in the extraction solvent has been suggested to prevent the binding of tertiary amines to non-specific binding sites on glassware and other surfaces (Gupta and Molnar, 1979). Indeed, the use of 0.05 M TEA was found to aid significantly in the extraction recovery of DPHM and [\(^2\)H_{10}]DPHM (Figure 12), while the use of silanized glassware did not result in a further increase in the extraction recovery of the compounds.

The validation of the analytical method involved estimation of intra- and inter-day variability. In addition, the method was cross-validated with a previously published method for the quantitation of DPHM (Yoo et al., 1986). The estimates of intra-day variability for DPHM and [\(^2\)H_{10}]DPHM were below 17% at 2.0 ng/mL, and below 8% at all other concentrations investigated in all three of the biological matrices tested (Table 1). The measured inter-day variability for the compounds was below 15% at 2.0 ng/mL, and below 10% for all other points (Table 2). These values fall within the acceptable guideline of ±20% at the LOQ and ±15% at other concentrations above the LOQ (Carr and Wahlich, 1990, and Shah et al., 1992).

Furthermore, the correlation between the concentrations measured using the GC-NPD method (Yoo et al., 1986) and the current method was excellent, suggesting that the two methods are highly comparable. The results of these validation experiments and the experiments detailing the sample stability during storage and sample work-up suggest that the method developed is robust, and measurements of DPHM and [\(^2\)H_{10}]DPHM concentrations in the biological matrices examined can be made with a high degree of confidence. The minimal quantifiable concentration or the LOQ of the previously published GC-NPD used in the quantitation of DPHM in biological fluids obtained from pregnant sheep was also 2.0 ng/mL; therefore, this method does not offer any advantage over the previously published method with regard to sensitivity (Yoo et al., 1986). Rather, the advantage of the current method is the ability to
simultaneously quantitate both DPHM and \([^{2}\text{H}_{10}]\text{DPHM}\) when present together in a biological sample during one chromatographic run.


A better understanding of the \textit{in vivo} metabolism of the drug can be obtained through the study of the resulting metabolites following administration of the intact drug. Of the metabolites identified to date, unconjugated and various conjugates of DPMA have been shown to be prominent urinary metabolites of DPHM in dogs, rhesus monkeys, and humans (Drach and Howell, 1968, Drach \textit{et al.}, 1970, Chang \textit{et al.}, 1974, Glazko \textit{et al.}, 1974, and Luo \textit{et al.}, 1991). Furthermore, unconjugated DPMA has also been detected in plasma and urine of adult sheep following the administration of DPHM, suggesting that a similar pathway is also functional in sheep. The availability of \([^{2}\text{H}_{10}]\text{DPHM}\) and an analytical method for the simultaneous quantitation of DPHM and \([^{2}\text{H}_{10}]\text{DPHM}\) has facilitated the application of stable isotope techniques to study the pharmacokinetics of DPHM in pregnant, non-pregnant, and fetal sheep. Thus, the development of an analytical method for the quantitation of DPMA and \([^{2}\text{H}_{10}]\text{DPMA}\) was pursued. Both DPMA and \([^{2}\text{H}_{10}]\text{DPMA}\) were synthesized and purified. In addition, a GC-MS/El analytical method using SIM was developed to simultaneously quantitate both \([^{2}\text{H}_{10}]\text{DPMA}\) and DPMA.

The derivatization of analytes for GC-MS quantitation can serve a variety of purposes. The derivatization of polar molecules can increase their volatility, and thus make them amenable to GC analysis. In addition, derivatization can improve instrument sensitivity for an analyte through improved chromatography and fragmentation characteristics during GC-MS analysis.
(Ahuja, 1976, and Pierce, 1993). Because DPMA and \([{}^{2}{H}_{10}]\)DPMA contain polar carboxylic acid functional groups, these analytes required derivatization. Two derivatives were tested during method development; namely, tert-butyldimethylsilyl (TBDMS) and pentafluorobenzyl (PFB) derivatives. Both types of derivatives of DPMA and \([{}^{2}{H}_{10}]\)DPMA resulted in good chromatography. The PFB derivatives did not offer any advantages over the TBDMS derivatives for GC-MS/EI quantitation and were more difficult to prepare; therefore, the TBDMS derivatives were utilized.

The tert-butyldimethylsilyl (TBDMS) derivatives of DPMA and \([{}^{2}{H}_{10}]\)DPMA underwent extensive fragmentation under EI conditions (70 eV), with the majority of fragments resulting from the breakage of the ether linkage of DPMA and \([{}^{2}{H}_{10}]\)DPMA (Figure 19). No molecular ions were detected, and the characteristic fragments \([M-57]^+ (i.e., the loss of the tert- butyl group) were present only in low abundance (Figure 19). Extensive fragmentation of the trimethylsilyl derivative of DPMA was also noted by Chang et al. (1974). Nevertheless, there were a number of fragment ions which retained the stable isotope label and were of good intensity for SIM quantitation of DPMA and \([{}^{2}{H}_{10}]\)DPMA \((m/z 167 and 183 - DPMA, and m/z 177 and 193 - \([{}^{2}{H}_{10}]\)DPMA). Initially, fragment ions \(m/z 183 and 193\) were chosen for the development of the analytical method. However, it was discovered that the fragmentation of DPMA also resulted in a small \(m/z 193\) fragment ion of unknown origin (Figure 19). This fragment resulted in chromatographic interference in the \(m/z 193\) ion chromatogram used to quantitate \([{}^{2}{H}_{10}]\)DPMA. Although fragment ions \(m/z 167\) and 177 gave good sensitivity and retained the stable isotope label, co-extracted components from adult urine co-eluted with DPMA in the \(m/z 167\) ion chromatogram, resulting in the inability to reliably quantitate DPMA in urine. As a result, the fragment ions \(m/z 183 and 177\) were chosen to quantitate DPMA and
[\textsuperscript{2}H\textsubscript{10}]DPMA in plasma and urine. Despite the fact that this choice of fragment ions was less optimal than fragment ions from the corresponding fragments of unlabeled and labeled metabolite (i.e., m/z 167 and 177, or 183 and 193), the assay method appeared to be free from complications resulting from this choice of ions (i.e., non-linearity etc.). In addition, intra-day variability studies conducted in plasma using ions m/z 167 and 177, 183 and 193, or 183 and 177 all gave similar results (unpublished results), suggesting that this choice of ions would be adequate for the quantitation of DPMA and [\textsuperscript{2}H\textsubscript{10}]DPMA. Furthermore, the ion chromatograms (i.e., m/z 183 and 177) were free from chromatographic interference due to the elution of endogenous components in ovine plasma and urine at the retention times of DPMA and [\textsuperscript{2}H\textsubscript{10}]DPMA (Figure 20). Thus, despite the inability to use corresponding fragment ions for DPMA and [\textsuperscript{2}H\textsubscript{10}]DPMA, fragment ions m/z 183 and 177 for SIM appeared to be suitable alternatives.

The extraction recovery of DPMA and [\textsuperscript{2}H\textsubscript{10}]DPMA from plasma appeared to be constant at different concentrations (i.e., 5.0, 50.0, and 500.0 ng/mL) of the analyte. However, while the extraction from plasma was constant, there was an unexplained concentration dependence of analyte recovery from urine. That is, the extraction recovery was 95 and 99\% at 5.0 ng/mL of DPMA and [\textsuperscript{2}H\textsubscript{10}]DPMA, respectively, while at higher concentrations the average recoveries for the compounds were 76\% and 74\% at 50.0 and 500.0 ng/mL, respectively. At the higher concentrations, the extraction recovery in urine and plasma were comparable. The reason for the higher recoveries from urine at the lower concentrations of DPMA and [\textsuperscript{2}H\textsubscript{10}]DPMA is not clear.

Poor sample stability either during storage and/or sample preparation can result in erroneous quantitation, which can lead to inaccurate interpretation of pharmacokinetic data. The stability of DPMA and [\textsuperscript{2}H\textsubscript{10}]DPMA was shown to be adequate in most circumstances; however,
the analyte was found to be labile in the presence of acid (Figure 23). The extraction conditions employed in this assay required the acidification of the plasma and urine biological matrices with 1.0 M hydrochloric acid. Caution must be used during the extraction procedure to ensure that excess hydrochloric acid is not added, and further, that the extraction procedure is conducted rapidly. For our study, the addition of 400 µL of 1.0 M HCl was found to provide an adequate reduction in pH for plasma and urine (pH < 2) without significant loss of DPMA and [²H₁₀]DPMA. Excess acid (> than 1.0 mL of 1.0 M HCl) was found to reduce the recovery of DPMA and [²H₁₀]DPMA and substantially increase the sample to sample variability. Stability studies conducted under an acidic environment, similar to that encountered during sample preparation, have shown that DPMA was stable during the course of an extraction. The measured half-lives for the degradation of DPMA in sample matrices would translate into the degradation of 1.0%, 0.6%, and 0.4% of DPMA in water, plasma, and urine during the 30 minute time interval required for the extraction procedure. Thus, despite the acid labile nature of DPMA, degradation during a rapid extraction (less than 30 minutes) using the extraction method outlined above would appear to only minimally contribute to a decrease in the recovery of DPMA and [²H₁₀]DPMA.

The results of the method validation experiments (i.e., intra-day and inter-day variability studies) and the analyte stability experiments suggest that the newly established GC-MS assay method is robust, and that the concentrations of DPMA and [²H₁₀]DPMA in ovine plasma and urine samples can be measured with a high degree of confidence.
4.3. Disposition of DPHM in Non-pregnant Sheep

The pharmacokinetic parameters obtained during studies examining the disposition of DPHM in non-pregnant sheep following a 100 mg IV bolus agree with the results obtained previously during a dose ranging study of the drug in non-pregnant sheep (Yoo et al., 1990). The clearance estimates in sheep (53 mL/min/kg) during the current study are greater than the clearances measured in humans (6-22 mL/min/kg) (Carruthers et al., 1978, Meredith et al., 1984, Blyden et al., 1986, and Simons et al., 1990). However, values for Vdss appear to be similar in both sheep (3.4 L/Kg) and humans (3.2-14.6 L/Kg) (Carruthers et al., 1978, Meredith et al., 1984, Blyden et al., 1986, and Simons et al., 1990). Unlike the previous studies examining DPHM disposition in non-pregnant sheep, this experiment also detailed the renal clearance of the drug, and its contribution to the total body clearance. The renal clearance of DPHM was 0.36 ± 0.28 mL/min/Kg, which corresponds to only ~0.3% of the total body clearance of the drug. The excretion of unchanged DPHM in the urine in other species, including humans (<4%), rhesus monkeys (~3%), dogs (~4%), rats (~1%), and rabbits (<3%) is greater than in sheep (Albert et al., 1975, Drach et al., 1970, and Parry and Calvert, 1982). These findings suggest that other non-renal routes play an important role in the elimination of DPHM from sheep. In the present study, cumulative bile collections were not possible; therefore, an accurate estimate of biliary secretion of unchanged DPHM could not be calculated. The concentrations of unchanged DPHM in the bile from the passive collections (for 10 minute intervals at 15, 30, 45, 60, 90, 120, 240, 360, and 720 minutes) were similar to the plasma concentrations of DPHM in the two animals in which bile was collected. Based on these concentrations and a literature value of the average bile flow in sheep (9.4 µL/min/Kg; Erlinger, 1982), a crude estimate of DPHM biliary secretion can be made. This estimate suggests that approximately 100 µg, or 0.1%, of the
administered dose is secreted as intact drug in the bile. Thus, it appears that neither renal nor biliary elimination of intact DPHM can account for a significant portion of the administered dose, and thus, the total body clearance in sheep. It is, therefore, likely that hepatic or extra-hepatic biotransformation of DPHM and/or non-reversible tissue binding plays a major role in DPHM elimination in this species.

4.4. Isotope Effect Studies

The application of stable isotope labeled drugs in analytical chemistry, pharmacology, pharmacokinetics, and drug metabolism has proven to be useful (Browne, 1986, Browne, 1990, Eichelbaum et al., 1982, Baillie, 1981, and Murphy and Sullivan, 1980). In pharmacokinetic studies, stable isotopically labeled drugs have often been co-administered with their unlabeled counterparts to assess both absolute and relative bioavailability, drug distribution, biotransformation and excretion, chrono-pharmacokinetics, dose-dependent pharmacokinetics, and drug-drug interactions (Browne, 1990, and Murphy and Sullivan, 1980). However, to successfully utilize a SIL drug in pharmacokinetic studies which involve the administration of both labeled and unlabeled drug, it is imperative that the SIL drug be "pharmacokinetically equivalent" to its unlabeled counterpart (Baillie, 1981). In effect, the labeled drug must share similar absorption, distribution, elimination, protein binding, and metabolism characteristics with the unlabeled drug (Van Langenhove, 1986, and Eichelbaum et al., 1982). Instances can arise where the disposition of the labeled drug can be different from that of the unlabeled drug; this is referred to as an "isotope effect" (Van Langenhove 1986). Use of deuterium labeled drug can result in two types of isotope effects, namely, a kinetic effect and/or a physicochemical effect. A chemical bond between a heavy isotope and another atom will be stronger than the equivalent
bond between the light isotope and that atom. If breaking this bond is a rate limiting step in the biotransformation and subsequent elimination of the compound, then the labeled drug may be eliminated slower than the unlabeled drug, yielding a kinetic isotope effect (Melander and Saunders, 1980). Physicochemical isotope effects can occur due to differences in pKa, lipid solubility, and possibly plasma protein binding of the labeled compound due to incorporation of deuterium (Van Langenhove, 1986). The presence of an in vivo “isotope effect” during a pharmacokinetic study involving the simultaneous administration of both labeled and unlabeled drug can seriously limit the usefulness of a SIL drug. For pharmacokinetic studies, isotope effects must either be absent or non-significant (i.e., within the error limits of the measurement method) (Wolen, 1986, Browne, 1990, VandenHeuvel, 1986, and Van Langhove, 1986). A common method for assessing the presence or absence of an isotope effect involves the simultaneous administration of equimolar doses of both labeled and unlabeled drug, and measurement of both the pharmacokinetic parameters to be studied and the concentrations of labeled and unlabeled drug in the appropriate biological fluids (plasma, urine, etc.). (Eichelbaum et al., 1982, Browne, 1990, and Baillie, 1981). Therefore, prior to utilizing $[^2H_{10}]$DPHM on a routine basis, isotope effect studies must be carried out.

Three studies were conducted, namely, adult bolus, fetal bolus, and fetal infusion, in order to rule out the presence of an isotope effect in the disposition of $[^2H_{10}]$DPHM. No differences were evident between DPHM and $[^2H_{10}]$DPHM plasma concentrations, AUC ratios, and pharmacokinetic parameters calculated during these experiments. The plasma data suggest that our experimental approach (the simultaneous administration of DPHM and $[^2H_{10}]$DPHM) can be used in pharmacokinetic studies conducted in the ovine fetal/maternal unit without the results being confounded by the presence of an isotope effect. However, an additional precaution
of alternating the route of administration of DPHM and $[^2\text{H}_{10}]$DPHM was taken in all studies except the simultaneous fetal/maternal infusions. This was to ensure that possible subtle differences which may not have been detected by the experiments conducted above would not bias our results. It was also demonstrated that no differences were detected between the concentrations of DPHM and $[^2\text{H}_{10}]$DPHM in adult ovine urine (adult bolus), fetal tracheal and amniotic fluids (fetal bolus studies), and fetal urine and amniotic fluid (fetal infusion studies). Furthermore, the analysis of DPMA and $[^2\text{H}_{10}]$DPMA in maternal plasma and urine (adult bolus) and in fetal plasma (fetal infusions) suggests that there is no isotope effect in the disposition of this metabolite. However, this must be independently confirmed via a simultaneous administration of both DPMA and $[^2\text{H}_{10}]$DPMA.

The pharmacokinetic parameter estimates for DPHM and $[^2\text{H}_{10}]$DPHM in the adult non-pregnant sheep correlate with the estimates reported by Yoo et al. (1990). However, following fetal bolus administration of intact drug, the estimates of $\alpha$ and $\beta$ are greater than the fetal parameters calculated following maternal bolus administration (Yoo et al., 1986). In addition, fetal CL$_T$ of DPHM following fetal administration (1.0 minute bolus and 90.0 minute infusion) is 2-3 fold larger than that following a fetal infusion to steady-state (Yoo et al., 1993). The “sink” conditions in the ewe (i.e., a large constant concentration gradient from fetus to mother) following a fetal bolus could lead to increased drug transfer from fetus to mother, and could be partially responsible for this observation. In the current study the weight corrected value for fetal CL$_T$ is almost 10 fold greater than the corresponding weight corrected estimate for CL$_T$ in adult sheep (Tables 5, 7, and 8). A similar observation has also been made for DPHM following fetal and maternal infusions to steady-state; however, the magnitude of the difference reported in this study is much greater than that previously reported (10 fold vs. ~3 fold) (Yoo et al., 1993).
addition, the Vdss in the fetus was also much larger than that observed in the ewe (22.3 ± 8.7 L/Kg fetal weight vs. 1.8 L/Kg maternal weight). The reasons for the large differences between the fetal and maternal weight corrected Vdss and CLT are presently not well understood. However, it is possible that the large absolute maternal volume of distribution and clearance may be contributing to the fetal estimate following fetal drug administration due to the rapid fetal to maternal transfer of DPHM across the placenta.

4.5. Hepatic First Pass Metabolism of DPHM in Adult and Fetal Sheep

4.5.1. Hepatic First Pass Metabolism of DPHM in Adult Sheep During Normoxia

In adult non-pregnant sheep, DPHM undergoes extensive first-pass metabolism following mesenteric venous administration, with only 6.8 ± 3.1% availability. This means that 93.2 ± 3.1% of the dose is eliminated prior to reaching the systemic circulation following mesenteric venous (portal) administration. From these data it appears that hepatic metabolism and/or hepatic uptake of DPHM is a major component of the total body clearance in non-pregnant ewes. Hepatic clearance (CLH) can be estimated using the equation CLH = Qh*E, where Qh is the hepatic blood flow and E is the extraction ratio across the liver (Gibaldi and Perrier, 1982, and George and Shand, 1982). Because the concentration of DPHM in ovine blood cells was not significantly different than the drug concentration in plasma (See Section 3.4.1), an estimate of total hepatic blood flow was used to calculate the hepatic clearance of DPHM (Gibaldi and Perrier, 1982). Using a literature value for hepatic blood flow in adult non-pregnant sheep (Qh) of 55 mL/min/Kg, the hepatic clearance of the drug is estimated to be ~51 mL/min/Kg (Katz and Bergmann, 1969). Since the measured total body clearance of DPHM is 65 mL/min/Kg,
approximately 80% of the total body clearance of this compound can likely be attributed to hepatic uptake or metabolism. This data would appear to confirm the results obtained earlier in non-pregnant sheep, where renal excretion of intact drug did not contribute significantly to the total body clearance. The availability of DPHM measured in sheep is lower than that reported in humans following oral administration (43-72%) (Albert et al. 1975, Carruthers et al., 1978, Spector et al., 1980, Meredith et al., 1984, and Blyden et al., 1986). However, in both species (humans and sheep) the hepatic clearance of the drug appears to contribute significantly to the observed total body clearance (Meredith et al., 1984). The current data (i.e., the high hepatic extraction ratio ~ 90% of DPHM) would suggest that the hepatic clearance of DPHM is high, or liver blood flow dependent ($CL_{H} = Q_{H}$) (Wilkinson and Shand, 1975). That is, it is likely that changes in liver blood flow could influence the hepatic clearance of DPHM following systemic administration.

### 4.5.2. Hepatic First-Pass Metabolism of DPHM in Adult Sheep during Mild Hypoxemia

Drug elimination is a function of the delivery of the substrate to the organ of metabolism and the capability of that organ to eliminate the drug. Hypoxia has been demonstrated to alter both of these functions (du Souich, 1978), and thus lead to changes in the pharmacokinetic parameters. The current study was undertaken in non-pregnant ewes to investigate the influence of acute mild to moderate hypoxemia on the pharmacokinetics and first-pass hepatic metabolism of DPHM. The infusion of $N_{2}$ gas into the lung via a tracheal catheter in non-pregnant sheep resulted in significant reductions in $P_{O_{2}}$ and $O_{2}$ saturation. All five animals experienced similar mean reductions in $P_{O_{2}}$ and $O_{2}$ saturation ($P_{O_{2}} 48.8 \pm 3.1 \%$ and $17.7 \pm 1.1 \%$ $O_{2}$ saturation) (Table 10). However, two ewes (1158 and 1154) experienced more severe initial reductions in
Po₂ and O₂ saturation (over the first 30 minutes) than the other three animals (60 vs. 40 % reduction in Po₂, and 30 vs. 10% reduction in O₂ saturation, respectively). Although there was a tendency for CLₜ and V₃ₕs to increase during hypoxemia, these differences were not statistically significant from values calculated during the normoxic period (Mann Whitney U-Test, p>0.05). The hepatic first-pass extraction of DPHM appeared to be extensive in three of the animals following mesenteric administration, as reported in the normoxic period. In the other two ewes a large increase in bioavailability was noted (i.e., a 70 and 220 % increase; Table 11). The reason for the disparity within this study is unclear at present; however, the two animals in which the bioavailability increased were also the two animals which showed the greatest initial decrease in Po₂ and O₂ saturation (ewes 1154 and 1158). Since DPHM appears to be a high hepatic clearance drug (Section 4.5.1.), changes in both blood flow and hepatic intrinsic clearance may influence the bioavailability, while only changes in hepatic blood flow will alter the systemic clearance (Wilkinson and Shand, 1975). Wilkinson and Shand (1975) have also pointed out that for a high clearance drug, a small decrease in the intrinsic clearance may result in a large increase in bioavailability, and that an increase in the hepatic blood flow will result only in a proportionate reduction in the bioavailability (i.e., F = Qₜ/(Qₜ + fₜₕ*CLₜₕt), where fₜₕ is the fraction unbound, and CLₜₕt is the intrinsic clearance of the free drug by the liver; Wilkinson, 1983). Thus, a reduction in hepatic intrinsic clearance of DPHM induced by the initial hypoxemic insult may partially explain the results obtained in the two animals in which large increases in the bioavailability were noted with no apparent changes in systemic clearance. The reason similar changes do not occur in the other three animals in this study is not clear, but may be due to initial reductions in Po₂ and O₂ saturation not reaching a critical threshold which would reduce the intrinsic clearance of DPHM. Such a phenomenon has been demonstrated for the
intrinsic clearance of propranolol in the isolated perfused rat liver. Reductions in hepatic oxygen delivery do not alter propranolol clearance until a threshold is reached, after which the propranolol clearance decreases in a linear fashion with a reduction in O₂ delivery (Elliot et al., 1993). Further, reductions in the total body clearance of lidocaine and indocyanine green (high clearance drugs) have been demonstrated in vivo with rabbits (Marleau et al., 1987). This reduction was speculated to be due to a reduction in the intrinsic clearance of both lidocaine and indocyanine green. However, in a subsequent study of mild to moderate hypoxia in dogs, a reduction in lidocaine clearance could not be shown (du Souich et al., 1992). It has been suggested that the threshold for decreasing the intrinsic clearance of various drugs appears to be highly species- and substrate-specific, and in some cases may even occur during mild hypoxia (Angus et al., 1990, and Jones et al., 1989). Thus, it appears that a reduction in the intrinsic clearance of DPHM due to the initial hypoxemic insult may partially explain the increase in bioavailability in the two animals with the largest initial reduction in Po₂ and O₂ saturation. However, there was also a lag period in achieving the maximum plasma concentrations of drug in the systemic circulation following mesenteric bolus administration in the two ewes most severely affected by hypoxemia (1154 and 1158). The reason for this is not clear. Changes in gut and portal venous blood flow due to redistribution of cardiac output do not appear to occur during mild to moderate hypoxia (du Souich et al., 1992). However, it is difficult to compare the current study to previous studies, because in the current study hypoxemia was induced much earlier (i.e., 15 minutes) relative to drug administration. Nesarajah et al. (1983) have demonstrated that changes in blood flow distribution can occur immediately following acute severe hypoxia, while changes in blood flow redistribution following prolonged mild to moderate hypoxia are uncommon. Therefore, it is possible that immediate changes in gut and liver blood
flow redistribution have influenced the bioavailability of DPHM in the current study. However, changes in hepatic tissue binding and or uptake/release of DPHM during the hypoxemic episode may also contribute to this phenomenon. Jones et al. (1984) demonstrated an apparent release of propranolol from isolated rat livers into the perfusion media following 20 minutes of severe hypoxia. Whether a similar phenomenon occurs with DPHM, resulting in a release of drug bound in the liver during mild to moderate hypoxemia in sheep, is presently not known. Thus, the exact reason for this increase in the bioavailability in two of the ewes in the current study is not known; however, it is possible that changes in hepatic intrinsic clearance, hepatic blood flow, and hepatic binding may all have been contributing factors.

4.5.3. Fetal Hepatic First-Pass Metabolism Following Umbilical Venous Administration

To examine the extent of the DPHM first-pass effect in the fetal lamb, we chose to employ simultaneous, paired injections of DPHM and [2H10]DPHM via the abdominal inferior cava (i.e., tarsal vein) and umbilical vein. The latter route was utilized rather than the mesenteric vein because of the large proportion of total hepatic flow supplied by the umbilical vein in the fetus (Edelstone et al., 1978, and Bristow et al., 1981), and because this is the route by which drugs reach the fetus from the mother.

Unlike the situation in adults, where the majority of hepatic blood flow comes from the portal vein (~80%) and the hepatic arteries (~20%) (Katz and Bergman, 1969), the umbilical vein provides a substantial additional vascular input to the fetal liver. This vessel supplies ~93% of the blood flowing to the left lobe and ~60% of the perfusion of the right and caudate lobes of the liver in the fetal lamb (Edelstone et al., 1978, Bristow et al., 1981, and Holzman, 1984). The umbilical
venous contribution to hepatic perfusion comprises ~50% of total umbilical flow (the remaining ~50% bypassing the liver via the ductus venosus) (Edelstone et al., 1978, Bristow et al., 1981, and Holzman, 1984) (Figure 52). Thus, if the fetal liver were functional in drug uptake and/or elimination, a portion of the drug in the umbilical venous blood which traverses the fetal liver could be removed in a fashion analogous to hepatic first-pass metabolism in adults following oral or intraperitoneal drug administration. Previous studies of lidocaine disposition in pregnant sheep have demonstrated the preferential distribution of drug present in umbilical venous blood to the fetal liver following maternal administration. It was shown that two minutes following a maternal bolus dose of the drug, the concentration in fetal liver was 7.5 fold greater than the fetal plasma level of lidocaine, whereas the drug levels in maternal plasma and liver tissue were similar (Finster et al., 1971). The difference between the ewe and fetus does not appear to be due to preferential affinity of the fetal liver for the drug, since a subsequent study found that lidocaine concentrations in fetal liver do not exceed those in plasma 4 hours following drug administration (Kennedy et al., 1990). In these studies of lidocaine, the impact of the initial fetal hepatic uptake of the drug on fetal systemic levels was not assessed. However, such a mechanism could function in minimizing fetal exposure to maternally-derived drugs.

An alternative approach to that employed in the current study to assess fetal hepatic drug uptake would be via the Fick method. This method would require the measurement of drug concentrations in fetal arterial and umbilical and hepatic venous blood coupled with measurement of liver blood flow. Due to the multiple vascular inputs to the fetal liver, this approach is more complicated than in the adult. However, it has been employed in fetal lambs in utero to study hepatic oxygen and carbohydrate utilization (Bristow et al., 1981), and in exteriorized fetuses to assess propranolol uptake by the liver (Marshall et al., 1981, and Mihaly et al. 1982, see below).
However, the Fick method involves extensive surgical preparation of the fetus, which is not a requirement with our approach.

**Fetal Liver (Dorsal View)**

![Diagram of fetal liver]

Figure 52: An anatomical sketch of the fetal liver in sheep. HV - hepatic vein, IVC - inferior vena cava, DV - ductus venosus, UV - umbilical vein.

Both bolus drug injection and infusion to steady-state were employed to assess fetal hepatic DPHM uptake. The former protocol was similar to that employed in the adult sheep. However, in marked contrast to the results in non-pregnant ewes, the availability of DPHM following umbilical administration in the fetus was $1.10 \pm 0.07$, a value not significantly different from 1. However, if enzymes capable of metabolizing DPHM are functional in the fetal liver, but present only in small quantities, the bolus administration of DPHM could result in a large amount of the drug being rapidly presented to the liver, leading to saturation of the enzymes and limited fetal hepatic metabolism of the drug. The simultaneous umbilical and tarsal venous infusions over 90 minutes were used to examine this possibility, as this would result in an approximately 100 fold lower
concentration of drug being delivered to the liver in umbilical venous blood (~0.15 mg/mL vs. ~12.5 mg/mL for the bolus 1 min injection, assuming an umbilical blood flow of 400 mL/min).

With the infusion protocol, the difference between the clearances calculated from the tarsal venous (control) and umbilical infusion (test) would give the net hepatic clearance of DPHM with the latter route of administration. However, the difference between the clearance values (28.7 ± 44.8 and -20.1 ± 53.9 mL/min/Kg for FA and CA values, respectively) were not significantly different from zero. As shown in Table 14, the inter-animal variation in the values was large. In two animals (E#1142 and 1242#1), the difference between umbilical and tarsal venous clearance values was consistently positive, suggesting hepatic DPHM uptake. However, in another ewe (E#2164), a consistent negative value was obtained, while for the other two there was no consistent difference.

The source of this large variability is not known; however, it could result from the subtraction of two large, variable estimates of fetal total body DPHM clearance to yield a much smaller value for "hepatic clearance" with a much larger degree of variability. Overall, the results of both experimental approaches indicate that the liver of the fetal lamb does not function effectively in the presystemic removal of DPHM from umbilical venous blood, and hence that the fetal equivalent of a hepatic first-pass effect cannot be demonstrated.

The lack of an obvious fetal first-pass effect for DPHM does not rule out the possibility that the fetal liver can metabolize the drug. One obvious complicating factor in the study design is that ~50% of umbilical venous blood bypasses the liver via the ductus venosus (Edelstone et al., 1978, and Rudolph, 1985), and so is not exposed to hepatic drug uptake. However, even with this shunt, if fetal hepatocellular metabolic capacity for DPHM were similar to that observed in the adult, we would expect to observe a systemic availability of ~60% (i.e., the ~50% of total drug administered by passing the liver through the ductus venosus, plus ~20% of the remaining drug that passes through
Thus, it appears that the ability of the fetal liver to metabolize the drug is far less than in the adult. However, data from in vitro studies of hepatic microsomes obtained from fetal lambs in late gestation indicate conjugation of morphine and acetaminophen at appreciable rates (Dvorchik et al., 1986, and Wang et al., 1986b). In addition, glucuronide conjugates of ritodrine, labetalol, and acetaminophen are formed by fetal lambs in utero, though with the former 2 drugs, the involvement of the fetal liver in the conjugation reactions has yet to be demonstrated (Yeleswaram et al., 1993, Wright et al., 1991, and Wang et al., 1986a). Other drug biotransformation reactions have also been demonstrated in fetal hepatic microsomal preparations, including the N-dealkylation of methadone and meperidine, and the hydroxylation of benzo[a]pyrene and hexobarbital. However, these occur at rates lower than observed in the adult sheep (Dvorchik et al., 1986). N-dealkylation and deamination reactions are involved in DPHM biotransformation in adults of other species (dog, human, and rhesus monkey) (Drach et al., 1970, Chang et al., 1974, and Glazko et al., 1974). If these pathways are not developed to the same degree in the fetal lamb, then this could explain the apparent low extraction of DPHM across the fetal liver. It is not certain how these in vitro results correlate with fetal hepatic elimination of drugs in vivo. However, Rane et al (1976) demonstrated that data from in vitro experiments (Km and Vmax) could be used to estimate the intrinsic clearance, and thus the hepatic first-pass metabolism of various drugs in vivo in adults. If a similar situation exists in the fetus, then the ~85% lower rate of N-demethylation of DPHM in fetal hepatic microsomes is certainly consistent with a lower first-pass effect for the drug in the fetus as compared to the adult (see Section 3.3.3.).

There appears to have been only one other attempt to assess fetal hepatic drug uptake. This study was conducted utilizing the Fick method to examine propranolol disposition in anaesthetized
fetal and adult sheep. In the adult, the drug was extensively extracted by the liver following portal venous administration (Marshall et al., 1981, and Mihaly et al., 1982). In contrast, with portal venous administration of the drug in the fetus, systemic availability was \( \sim 1 \) (Marshall et al., 1981), a result similar to that which we observed with umbilical venous administration of DPHM. With maternal infusion of propranolol, and thus delivery of the drug to the fetus via the umbilical vein, fetal hepatic extraction was \( \sim 30\% \) (Mihaly et al., 1982). However, the fetal right hepatic vein was sampled in these studies, and since the umbilical vein supplies only \( \sim 60\% \) of the flow to the right lobe of the liver (Bristow et al., 1981), the observed extraction could have at least, in part, been due to a dilutional effect resulting from the mixing of umbilical venous blood (high drug concentration) with blood from the portal vein (low drug concentration) in the right lobe of the liver. Also, the acute nature of these experiments could have affected fetal hepatic blood flow distribution via anaesthetic effects or exteriorization of the fetus. More studies of fetal hepatic drug uptake and metabolism are clearly warranted.

Oxygen saturation and the concentrations of nutrients and endogenous metabolites have been noted to be higher in the carotid arterial (CA) blood than in femoral arterial (FA) blood (Charlton and Johengen, 1984, and Rudolph et al., 1991). A similar finding was also apparent in the current study, in which statistically significant differences between FA and CA glucose and lactate concentrations, oxygen saturation, and oxygen partial pressure were noted. All were greater in CA blood as compared to FA blood (See Table 15). The explanation for these observations primarily involves two factors: 1) the mixing in the thoracic vena cava of umbilical venous blood passing through the ductus venosus and inferior vena caval blood from the lower body, which results in concentrations of \( O_2 \) that are substantially higher than in superior vena caval blood; and 2) there is preferential shunting of inferior vena caval blood through the
foramen ovale to the left heart, and therefore, a lesser degree of dilution of this blood with
deoxygenated pulmonary venous blood than is the case in the right heart, where the more highly
oxygenated inferior vena caval blood mixes with the superior vena caval return (Dawes, 1968,
Rudolph, 1985, and Teitel et al., 1982). An additional factor may be the incomplete mixing of
ductus venosus blood with abdominal inferior caval blood in the thoracic vena cava, and
preferential streaming of the former through the ductus venous. Evidence for this phenomenon
has come from studies demonstrating the preferential distribution to the fetal upper body of
radioactive microspheres injected via the umbilical vein (Edelstone and Rudolph, 1979). It has
been suggested that a similar distributional phenomenon may occur with drugs infused directly
into the fetus (Rudolph, 1985). If this were to occur with maternally derived blood (i.e., via the
umbilical vein), there could be increased delivery of drug to the fetal heart and brain. This would
be particularly significant for agents which affect CNS or cardiac function. Differences between
FA and CA plasma concentrations of DPHM were not apparent following bolus administration;
however, this is not unexpected since rapid mixing of blood occurs due to rapid fetal circulation
times (2-4 seconds) (Power and Longo, 1975). However, a statistically significant difference was
noted between the FA and CA plasma concentrations of DPHM following tarsal venous and
umbilical venous infusions. Unlike the situation with O₂, Po₂, glucose, and lactate, the drug
level in the descending aorta was higher both with tarsal and umbilical venous infusions
(although with the latter route of infusion the concentration difference between FA and CA was
less than the former route). An explanation for these results may involve the fact that the higher
O₂ concentration in CA blood appears primarily due to the lesser degree of dilution of inferior
vena caval blood in the left heart, and hence, ascending aorta compared to the right heart and
descending aorta. In the fetal lamb, inferior vena caval blood comprises ~77% of venous return
to the left heart, with the remainder being supplied by the lungs and coronary circulation, whereas in the right heart ~66% of venous return in supplied by the inferior vena cava, with the rest coming from the upper body via the superior vena cava (Teitel et al., 1982). Thus, the concentration of O₂, glucose, and other placentally derived substances of intermediary metabolism in the inferior vena cava are diluted to a lesser degree in the left heart, resulting in higher concentrations in the ascending aorta than in the right heart and descending aorta (~90% of right ventricular output is delivered into the descending aorta via the ductus arteriosus, Rudolph, 1985, Teitel et al., 1982). With DPHM at steady-state, there would likely be minimal net uptake of drug by the tissues of the upper body, particularly by the skin, muscle, and bone. Thus, DPHM levels in the superior vena caval blood should not be much different from those in arterial blood. Therefore, there should be minimal dilution of the drug delivered to the right heart via the inferior vena cava. However, we have previously found that DPHM is taken up by the lung in the fetal lamb, and hence the pulmonary venous drug concentration is lower than in the pulmonary artery (Rurak et al., 1991). Therefore, in the left heart there would be mixing of inferior vena caval blood with pulmonary venous blood that had a lower drug concentration. The net result would be higher DPHM levels in descending aorta compared to the ascending aorta, which is the result obtained. The observation for a lower FA-CA DPHM concentration difference with umbilical venous infusions could be due to the preferential streaming of ductus venosus blood to the left heart and upper body, thereby counteracting the dilutional effect of the mixing of pulmonary venous and inferior vena cava blood in the left heart.

4.6. **Paired Maternal/Fetal Infusions of DPHM and [²H₁₀]DPHM**

4.6.1. **Fetal Behavioral Effects Following Simultaneous Maternal/Fetal Infusions of DPHM and [²H₁₀]DPHM**
There appear to be dose-related differences in the pattern of adverse effects reported with DPHM in humans. At lower circulating levels of DPHM (40-80 ng/mL) CNS depression has been reported, while at higher drug levels (6-22 X normal) CNS stimulation was observed (Douglas, 1985). Although a direct comparison of the effects of DPHM in the post- and the prenatal period is complicated by differences in the sleep pattern, the constellation of the drug effects of DPHM in the fetal lamb also appear to vary with plasma concentrations of the drug (Rurak et al., 1988). At low fetal plasma levels following maternal infusion of DPHM (i.e., 36.3 ng/mL) there are sedative-like effects (a reduction in low voltage ECoG and a decrease in fetal breathing movements), while at higher concentrations (i.e., 448 ng/mL) there was a decline in the amount of low voltage ECoG accompanied by a marked increase in the intermediate ECoG voltage pattern. In addition, initial transient declines in fetal arterial Po2 and pH associated with tachycardia and vigorous fetal breathing were noted (Rurak et al., 1988). The effects observed in the current study appear to be in between the effects noted in the previous studies. There was a marked increase in the intermediate ECoG voltage pattern, similar to that following fetal drug infusion in the previous study (Figure 45), accompanied by a tendency for fetal breathing movements to decrease during the infusion (not statistically significant), similar to the results obtained from the previous maternal infusion of DPHM (Figure 46). The “intermediate” nature of the fetal effects in the present study may be due to the plasma concentrations of DPHM [282.4 ng/mL (238.2 ng/mL [2H10]DPHM + 45.1 ng/mL DPHM)], which fall between the two plasma concentration ranges from the previous study. These data, taken with the previous results, suggest that there may be a relationship between the plasma concentration of DPHM and the constellation of fetal effects observed. However, whether there is a clear pharmacodynamic
relationship between fetal behavioral effects and fetal DPHM plasma concentrations requires further study, preferably including measurement of brain tissue or extracellular fluid drug levels.

In a previous study, a transient increase in the fetal heart rate was noted immediately following the start of a fetal infusion of DPHM. This phenomenon was attributed to the anticholinergic effects of DPHM observed at higher doses (Rurak et al., 1988, and Douglas, 1985). A similar finding was not observed in the present study; rather, a decrease in the fetal heart rate during the course of the fetal-maternal infusions was noted. The reasons for this are not presently clear, but again it may relate to differences in the fetal circulating level of the drug, particularly during the initial infusion period.

4.6.2. Trans- and Non-Placental Clearances of DPHM in the Maternal/Fetal Unit.

In the current study there was a tendency for the mean fetal steady-state plasma concentrations to be less than those observed by Yoo et al. (1993), despite the same fetal infusion rates. As a result, the clearance estimates based on the fetal steady-state arterial plasma concentrations appeared to be greater than those estimated by Yoo et al. (1993) (Table 26).
Table 26: Comparison between total, trans-, and non-placental clearances from a previous study (Yoo et al., 1993) using time separated maternal and fetal infusions, and the current study using simultaneous fetal and maternal infusions of $[^{2}H_{10}]$DPHM and DPHM.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Yoo et al. (1993)</th>
<th>Current Study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mL/min/Kg)</td>
<td>(mL/min/Kg)</td>
</tr>
<tr>
<td>CLmm&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.3 ± 9.8</td>
<td>40.0 ± 5.2</td>
</tr>
<tr>
<td>CLff</td>
<td>223.9 ± 95.8</td>
<td>356.0 ± 106.1</td>
</tr>
<tr>
<td>CLmf</td>
<td>41.0 ± 24.1</td>
<td>54.0 ± 32.4</td>
</tr>
<tr>
<td>CLfm</td>
<td>124.4 ± 60.9</td>
<td>221.0 ± 71.6</td>
</tr>
<tr>
<td>CLmo&lt;sup&gt;a&lt;/sup&gt;</td>
<td>38.0 ± 4.5</td>
<td>43.2 ± 9.5</td>
</tr>
<tr>
<td>CLfo</td>
<td>99.5 ± 36.8</td>
<td>135.0 ± 59.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> - calculated based on maternal weight

Values are shown as mean (± S.D.) for comparison purposes.

A statistical comparison between the two estimates is difficult due to the large inter-animal variability in both the steady-state plasma concentrations and the resulting estimates of the non- and trans-placental clearances. The sample size required to show a statistically significant difference between two means of a predetermined magnitude (e.g., 10, 20, etc %) associated with the current level of variability can be calculated (Zar, 1984). To detect a 10% statistically significant difference between the clearance values calculated in the present study to those calculated by Yoo et al. (1993) would require a sample size of 105 animals for CLmm and 392 animals for CLff. Therefore, with only 5 the animals in the current study, no meaningful conclusions can be drawn from the differences between our present data and that of Yoo et al. (1993).

In the previous study of Yoo et al. (1993), the wash-out period between the time separated infusions was 48 hours, due to the short half-life of DPHM in the ovine maternal/fetal unit (i.e., ~ 60 minutes). Since the fetus grows at a rate of ~3-5% per day (Kong et al., 1975), it
is unlikely that fetal growth and differentiation of fetal organ systems during the wash-out period would result in detectable differences in DPHM disposition between the maternal and fetal infusions. However, this may not be the case for drugs which have a long disposition half-life, where in the course of a longer wash-out period between the time separated fetal and maternal infusions, detectable time dependent changes in drug disposition could occur, necessitating the use of simultaneous maternal/fetal infusions. In addition, simultaneous maternal and fetal infusions should provide more precise (less variable) estimates of the placental and non-placental clearances compared to time-separated infusions due to the elimination time dependent effects of fetal growth and development, and to a reduction in the inter-day variability within a maternal/fetal pair. In the present study there was a trend (though not statistically significant; F-test p>0.05) for a reduction in the variability of the clearance estimates CLmm and CLmo.

Simultaneous infusions of labeled and unlabeled drug offer additional benefits in that the likelihood of successful experiments is substantially increased (two separate experiments conducted at two different times on the same animal are not required). In addition, such paired experiments allow for a greater experimental utilization of the pregnant animals, since more experiments (following the appropriate wash-out period) can be conducted with a preparation over the 1-3 week experimental window available in each animal.

The trans- and non-placental clearances for the maternal-fetal unit are calculated by equations derived from a 2 compartment-open model (Figure 6). The assumptions of this model are that transfer and elimination must be first-order processes, no drug must be present at time infinity (i.e., drug must be entirely eliminated by CLmo and/or CLfo), and elimination and transfer occur from the central compartment in both mother and fetus. The approach used by Szeto et al. (1982) and Szeto (1982) to arrive at solutions for the trans- and non-placental
clearances of DPHM require that steady-state plasma concentrations be achieved, so that there is no net flux between drug present in the central and the peripheral compartments in mother and fetus. Another approach, which retains the same model structure as the Szeto approach but does not require steady-state plasma concentrations to be achieved, is the mass balance approach (Ebling and Jusko, 1986). The assumptions for the 2 compartment-open model using the mass balance approach are similar to those above, but in addition, it is assumed that elimination of drug does not occur from both maternal and fetal distributional compartments radiating off the central compartments (Ebling and Jusko, 1986). This model is “model-independent” with regard to tissue distribution, thus, the clearance estimates obtained from this model cannot provide information about the drug in the peripheral compartments. The estimates obtained from this approach correlate well with the estimates obtained from the Szeto approach (Table 19 and 20). With both approaches, measurable quantities of drug must be present in maternal plasma following fetal administration, and vice versa. If an infusion, rather than a bolus, is required to achieve measurable drug concentrations when using the mass balance approach, post-infusion sampling required to calculate the AUCs prolongs the experiment, and increases the total volume of fetal blood sampled. This would limit the usefulness of this approach.

A potential limitation of the 2 compartment-open model for fetal/maternal drug disposition is that it neglects the placenta as an organ of drug elimination. Rather, the placenta is considered to be only an organ of maternal-fetal exchange. If drug elimination did occur in the maternal and/or fetal compartment of the placenta, this would be calculated in the estimates of CLmo and CLfo, respectively (Szeto et al., 1982). We tested for placental DPHM metabolism utilizing the Fick method to estimate trans-placental clearance values. Traditionally, the Fick method has only been used to measure the uni-directional clearance of substances from fetus to
ewe (Wang et al., 1986a). However, with simultaneous infusions of DPHM and $[^2\text{H}]_{10}$DPHM, bi-directional placental clearances can be calculated. In the two animals, where both umbilical venous blood samples were collected and the umbilical blood flow was measured, the model independent estimates of both CLfm and CLmf were calculated following simultaneous fetal/maternal infusions of DPHM and $[^2\text{H}]_{10}$DPHM. The placental extraction of DPHM and $[^2\text{H}]_{10}$DPHM across the placenta measured in this experiment was similar to an estimate obtained by Yoo et al. (1989) (Table 21). The placental clearance estimates for CLmf were approximately 30% lower than the model derived estimates. While the estimates of CLfm were in good agreement for ewe 2181, in ewe 122Z the Fick derived estimate underestimated the model derived parameter by a factor of two. However, based solely on the mean extraction ratios from this experiment and that of Yoo et al. (1989), and the mean umbilical blood flow obtained in this experiment, estimates for CLmf (67.4 mL/min/Kg) and CLfm (168.4 mL/min/Kg) were calculated (Table 21). These estimates correlate well with the CLmf, but underestimate the CLfm by 24% in our study. A proportion of the difference between the model derived and the Fick estimates of trans-placental clearance may result from placentally derived blood mixing with blood returning from the fetal membranes (~6% of total umbilical blood, Makowski et al., 1968a) in the umbilical vein. Furthermore, this difference is unlikely to be due to placental metabolism of the drug. Placental DPHM metabolism (on the fetal side of the placenta) should result in a higher Fick estimate of CLfm, since the estimate would be determined in part by a value of umbilical DPHM extraction that included drug loss due to both fetal to maternal transfer and placental metabolism. Therefore, as discussed above, with the model derived estimates of fetal DPHM clearances, placental drug metabolism would be included in the estimate of CLfo. Overall, it appears the model independent estimates of trans-placental clearances, obtained by the Fick
method, are similar to those obtained with the 2 compartment-open model. Thus, significant placental metabolism of DPHM in pregnant sheep appears unlikely. In a previous study, the placental clearance of acetaminophen estimated using the Fick method was also found to correlate with the clearance values calculated using the 2 compartment-open model (Wang et al., 1986a). This may be significant because the magnitude of the fetal placental and non-placental clearances of DPHM (CLfm 221 mL/min/Kg and CLfo 135 mL/min/Kg) are substantially different from acetaminophen (CLfm 31 mL/min/Kg and CLfo 11 mL/min/Kg) (Wang et al., 1986a). Thus, the 2 compartment-open model appears to provide good estimates for at least two drugs which show quite different disposition characteristics, and it also suggests that the metabolism of both drugs by the placenta is minimal.

The model derived estimates of CLfm can also be compared to empirically derived estimates of CLfm. The fraction of the fetal dose ([2H10]DPHM) which is required to produce maternal steady-state plasma concentrations of the compound can be calculated from the maternal total body clearance (CLmm) and the maternal steady-state plasma concentrations of [2H10]DPHM (fraction of fetal dose = CLmm * Cbss_{maternal} [2H10]DPHM / K_0_{fetal} [2H10]DPHM). If the model derived estimates for CLfm are accurate, then the fraction of drug transferred across the placenta from the fetus to the mother (CLfm*AUCff/(CLfm + CLfo)*AUCff) estimated using model derived estimates of CLfm and CLfo should equal the empirical estimates of the fraction of the dose required to produce the observed maternal steady-state plasma concentrations of [2H10]DPHM. The model derived estimate (69 ± 11%) correlates well with the empirical estimate (70 ±7%). This evidence appears to further substantiate the validity of the 2 compartment-open model for DPHM disposition in the fetal/maternal unit.
Previously, a significant difference between the model derived estimates of CLfm and CLmf of DPHM was noted, with CLfm being three fold greater than CLmf. A similar finding was observed in the current investigation, with the difference between the model derived estimates for CLfm and CLmf being approximately four fold higher when estimated using the 2 compartment-open model. A similar difference was noted when calculations for numerous other drugs including morphine, methadone, and metoclopramide were made using this model (Szeto et al., 1982b, and Riggs et al., 1990). This phenomenon does not appear to be due to model introduced bias, since the difference between CLfm and CLmf calculated using the model independent method (i.e., the Fick method) was 4.0 and 2.2 fold in ewes 122Z and 2181, respectively. Yoo et al. (1993) demonstrated, for various drugs studied, that the magnitude of the difference between CLfm and CLmf correlated with the value of CLfm of the drug. The reason for this observation is not clear. However, more studies using drugs with varied disposition characteristics are required to examine this phenomenon.

The apparent volume of distribution of DPHM following fetal drug administration (fetal bolus or fetal infusion) is very large (Tables 12, 13, and 18), and can exceed the maternal weight corrected volume of distribution by 12-13 fold. It is clear that, despite the differences in the body composition between the fetal and maternal sheep, these apparent volumes of distribution are overestimated. The reason for this overestimation is not entirely clear, but may be due to the apparent volume of distribution measured following fetal drug administration being composed of both the fetal apparent volume of distribution and a maternal component. By definition, the apparent volume of distribution of is “a proportionality constant relating drug concentration in the blood or plasma to the amount of drug in the body” (Gibaldi and Perrier, 1982). It is clear that the amount of drug in the fetal body at any particular time does not correlate well with the
dose administered, since a large portion of the dose administered to the fetus is transferred across the placenta to the mother (~60-70% of the fetal dose in the case of DPHM). If the volume of distribution is corrected for this loss across the placenta (CLmm*AUCfm), the volume of distribution is reduced by ~ 66% (i.e., from 27.1 L/Kg to 9.2 L/Kg). In order for this corrected volume of distribution to be valid, negligible placental metabolism must be assumed; as discussed above, this assumption is probably valid for DPHM. This postulated mechanism for the higher \( V_{dss} \) estimates for DPHM in the fetus is supported by the results obtained with other drugs. For example, the apparent volume of distribution of ritodrine, a drug with poor placental permeability (AUC fetal/AUC maternal = 0.03) is not statistically different in the mother (10.1 L/Kg) and the fetus (8.5 L/Kg) following maternal and fetal bolus administrations, respectively (Wright, 1992). Labetalol, a drug with a placental permeability greater than ritodrine, but less than DPHM (AUC fetal/AUC maternal = 0.13), has a greater fetal than maternal volume of distribution (14.3 L/Kg vs. 3.0 L/Kg, respectively). The magnitude of the difference between the fetal and maternal \( V_{dss} \) of labetalol is larger than that of ritodrine, but less than DPHM (Yeleswaram, 1992). From these limited data, it appears that placental permeability may play a role in the fetal volume of distribution measured following fetal drug administration. The fetal weight corrected \( V_{dss} \) following correction for the proportion of the dose which is lost for the fetus to the mother is also substantially larger than the weight corrected estimate in the ewe. If the placenta is included (0.3-0.4 Kg), the weight corrected fetal \( V_{dss} \) is further reduced.

Although the placenta in terms of mass is relatively small, binding of DPHM to the placenta may be substantial. This is so because in the human and other species, the placenta has been shown to be rich in monoamine oxidase-type A (MAO-A) (Tan et al., 1991). The concentration of MAO-A in human placenta is almost two-fold greater than in the brain. (O'Carroll et al, 1989).
Furthermore, DPHM has been shown to bind extensively to MAO in rat lung and rat lung mitochondria (Yoshida et al., 1989, and 1990). If the binding of DPHM to MAO in the ovine placenta is similar to that reported in rat lung, the placenta may contribute significantly to the estimates of fetal Vdss.

As in the studies employing simultaneous 90 minute fetal infusions of labeled and unlabeled DPHM (section 4.5.3), drug concentrations were measured in both the fetal carotid and femoral arteries during the paired maternal-fetal drug infusions to check for preferential drug distribution to the upper body. As in the earlier experiments, the reverse situation was found with $[^{2}\text{H}_{10}]$DPHMT, the form of drug infused via the fetal tarsal vein. The mean FA-CA concentration difference of $6.7 \pm 2.8 \text{ ng/mL}$ was significantly different from 0, and similar to the value obtained in the 90 min infusion studies ($8.5 \pm 2.1 \text{ ng/mL}$). However, with DPHM (the drug administered to the ewe, and hence reaching the fetus via the umbilical vein) the FA-CA gradient of $1.1 \pm 1.1 \text{ ng/mL}$ was not significantly different from 0. This is different from the results obtained with umbilical venous drug infusion (where there was a positive FA-CA difference), although this was significantly lower than the value obtained with DPHM infusion via the tarsal vein (section 4.5.3).

As discussed previously, the higher FA drug concentration with inferior vena caval administration may be due to a minimal dilution of the drug delivered to the right heart in the inferior vena cava (via mixing with superior vena caval blood), compared to the situation with oxygen and other metabolic substrates which have lower concentrations in the blood returning from fetal tissues. In contrast, in the left heart there would be mixing of inferior vena caval blood with pulmonary venous blood that had a lower drug concentration, thereby resulting in higher $[^{2}\text{H}_{10}]$DPHM levels in descending aorta compared to the ascending aorta. The lack of a significant FA-CA DPHM concentration difference for maternally infused DPHM could be due to a greater degree of
preferential streaming of ductus venosus blood to the left heart than occurred during the 90 minute
umbilical drug infusions (section 4.5.3). The former result is most relevant to the normal situation
with fetal drug exposure, i.e., maternally administered drug reaching the fetus via the umbilical
vein. The findings suggest that preferential distribution of DPHM to the fetal brain, heart and other
upper body structures does not occur. However, studies of other drugs are warranted to determine
whether the results obtained with DPHM are generally applicable to a range of xenobiotics. In this
regard it would be interesting to study drugs, such as valproic acid, which do not accumulate in
fetal lung fluid and are probably not taken up by the lung (unpublished results; Mr. John Gordon).
This would allow additional proof for the proposed mechanism outlined above for the positive FA-
CA DPHM concentration difference, since according to this mechanism we would expect a
minimal or no difference between the FA and CA concentrations of a drug not subject to significant
uptake by the fetal lung.

4.6.3. Fetal and Maternal Renal Clearances of $[^2\text{H}_{10}]$DPHM and DPHM

The renal elimination of drugs and other substances is a function of three processes:
glomerular filtration, renal secretion, and tubular reabsorption (Roland and Tozer, 1989).
Glomerular filtration involves the filtration of plasma water and its dissolved components (<4
nm) across the capillary endothelium and the tubular epithelium (Ganong, 1985). Thus, for the
most part it is the free or unbound drug in plasma which is filtered by the glomerulus (i.e.,
$\text{CL}_{\text{renal}} = \text{fub} \times \text{GFR}$, where fub is the fraction unbound in plasma and GFR is the glomerular
filtration rate). If renal clearance exceeds the predicted clearance based on the filtration of the
unbound drug, active tubular renal secretion of the solute is inferred (Roland and Tozer, 1989).
Specific active renal secretion pathways exist for both acidic and basic compounds (Ganong,
Finally, the solute or drug in urine can be reabsorbed from the renal tubule back into the blood (Roland and Tozer, 1989).

The renal clearance of DPHM in the pregnant ewe is $0.012 \pm 0.009 \text{ mL/min/Kg}$. This value is significantly and markedly less than the reported glomerular filtration rate (GFR) in sheep, which is reported to be $2.4 \text{ mL/min/Kg}$ (Hill and Lumbers, 1988). This suggests that a portion of filtered and/or the secreted load of DPHM (based on plasma protein binding of 86% in sheep; Yoo et al., 1993) is reabsorbed in the renal tubule. The renal clearance of DPHM in adult sheep contributes less than 0.1% to total maternal body clearance of the drug. This is similar to the findings following bolus administration (Section 3.3.1.). In contrast, the fetal renal excretion of DPHM is $2.44 \text{ mL/min/Kg}$, which exceeds literature values for fetal GFR ($1.03 \pm \text{ mL/min/Kg}$) (Hill and Lumbers, 1988). This suggests that DPHM is being filtered and secreted by the kidney of the fetal lamb in late gestation. Our observations for the fetal renal clearance of DPHM are similar to previous studies conducted with other organic cations such as cimetidine, ranitidine, meperidine, and tetraethylammonium (Mihaly et al., 1983, Czuba et al., 1990, Szeto et al., 1979, and Elbourne et al., 1990). Meperidine has a renal clearance of $9.3 \pm 2.2 \text{ mL/min}$, which exceeds the rate of inulin clearance (i.e., $3.3 \text{ mL/min} \pm 1.4 \text{ mL/min}$), inferring that meperidine undergoes renal secretion (Szeto et al., 1979). Likewise, the renal excretion of cimetidine and ranitidine in fetuses at 140 days gestation also exceeded literature values for GFR (i.e., $12 \text{ mL/min}$ and $7.8 \text{ mL/min}$ for cimetidine and ranitidine, respectively) (Mihaly et al., 1983, and Czuba et al., 1990). In contrast, the renal clearance of lidocaine is low (i.e., $1.5 \text{ mL/min/Kg}$) and is below the reported renal glomerular filtration rate (Morishima, et al., 1979). Thus, the indirect evidence for most of the basic drugs examined thus far (meperidine, cimetidine,
ranitidine, and DPHM) suggests that renal secretion of organic cations is functional in the late gestational fetal lamb.

The reason for the discrepancy between the maternal and the fetal renal clearances of DPHM is not yet known. However, renal reabsorption may play a role in accounting for the greater renal clearance of DPHM in the fetal lamb than that in the mother. The pH of the fetal urine is ~ 6.78, compared to the pH in the maternal urine of 7.62. Because DPHM is a weakly basic drug with a pKa of 9.0, the fraction of the compound ionized fetal urine will be greater than in maternal urine, 99.4% vs. 95.6%, respectively. The lesser degree of ionization in the maternal urine will facilitate greater renal reabsorption of DPHM in the mother compared to the fetus, resulting in a greater amount of the drug excreted in the fetal urine (Roland and Tozer, 1989, and Ganong, 1983). In addition, the greater urine flow in the fetus (~14.2 mL/hr/Kg, Table 23) compared to the mother (~0.8 mL/hr/Kg) may also facilitate the increased fetal renal clearance of DPHM by reducing the reabsorption of the drug from the urine (Roland and Tozer, 1989).

4.6.4. Disposition of DPMA and [\textsuperscript{2}H\textsubscript{10}]DPMA in the Ovine Fetal/Maternal Unit following Simultaneous Maternal/Fetal Infusions

In humans, monkeys, and dogs, DPHM is thought to be metabolized via two sequential N-demethylation steps, followed by deamination (See section 1.7.3). The deaminated metabolite, DPMA, and its conjugated counterpart (glycine or glutamate conjugates) are major urinary metabolites in several species (Chang et al., 1974, Drach and Howell, 1968, Drach and Howell, 1970A, Drach et al., 1970, and Glazko et al., 1974). DPMA was present in the urine and plasma of non-pregnant ewes following DPHM administration (Section 3.3.3.2.1.). DPMA and [\textsuperscript{2}H\textsubscript{10}]DPMA were also present in both maternal and fetal plasma following the simultaneous
maternal and fetal infusions of DPHM and [\textsuperscript{2}H\textsubscript{10}]DPHM, respectively (Figure 41 and 42). It is important to remember that following the maternal administration of drug, the presence of metabolites in the fetal circulation may not be due to fetal drug metabolism, but could result from maternally derived metabolites which traverse the placenta (Wang et al, 1985). However, in the present study the formation of DPMA appears to occur in both mother and fetus. During the simultaneous infusion of DPHM and [\textsuperscript{2}H\textsubscript{10}]DPHM to mother and fetus, respectively, the concentration of DPMA is greater in the maternal than in the fetal arterial plasma, while the concentration of [\textsuperscript{2}H\textsubscript{10}]DPMA is higher in the fetal than in maternal arterial plasma. If this metabolite were formed by the mother and transferred to the fetus, the ratio of DPMA to [\textsuperscript{2}H\textsubscript{10}]DPMA in fetal plasma should be similar to the ratio in maternal plasma; this is clearly not the case (Figure 41 and 42). Furthermore, it is unlikely that [\textsuperscript{2}H\textsubscript{10}]DPMA is selectively transferred from maternal plasma to fetal plasma because this would require a 20 fold greater uptake of [\textsuperscript{2}H\textsubscript{10}]DPMA, compared to DPMA. This is not likely to occur since no isotope effect was evident from the plasma concentrations of DPMA and [\textsuperscript{2}H\textsubscript{10}]DPMA following simultaneous fetal infusions of DPHM and [\textsuperscript{2}H\textsubscript{10}]DPHM (Figure 35). Moreover, as discussed below, the available data suggests limited placental permeability of DPMA in sheep. Thus, our data strongly suggests that the metabolism of DPHM to DPMA occurs in the fetus as well as in the mother. The source of this metabolite in the fetus and mother and its quantitative importance are currently unknown.

The peak plasma concentrations of DPMA and [\textsuperscript{2}H\textsubscript{10}]DPMA occur much later in the fetal plasma in comparison to the maternal plasma. This may be a function of the longer apparent elimination half-life of DPMA in the fetus (911.1 ± 151.9 min) compared to the mother (180.4 ± 13.0 min)(Houston, 1982). The elimination rate of the metabolite was calculated based on a 1
compartment model due to constraints imposed by the sparse data (Figure 7). If the metabolite displays 2 or more exponential disposition phases, the model derived estimate of the elimination rate will likely be overestimated (T$_{1/2}$ will be underestimated). Therefore, these values must be considered to be "apparent" estimates. The apparent elimination half-life of the metabolite is substantially greater than that of the parent drug, which in mother and fetus are 70.5 ± 6.9 and 51.8 ± 7.2 minutes, respectively. In most cases, drug metabolism forms more water soluble metabolites, and thus biotransformation expedites the removal of the drug and the resulting metabolites from the body. However, the formation of DPMA appears to impede the elimination of the DPHM body load (i.e., DPHM and metabolites). The extended half-life of DPMA in the fetal plasma, compared to the maternal plasma, suggests that the elimination pathways for this metabolite are not as developed in the fetus as they are in the ewe (see Section 4.6.5.). Furthermore, the persistence of DPMA in the fetal plasma suggests that DPMA does not readily transfer across the placenta from fetal blood to maternal blood, compared to the parent drug (DPHM). The possibility of minimal placental transfer is further substantiated by the zero extraction of DPMA by the placenta in the two ewes with UV catheters. However, the larger ratio of \text{AUC([DPMAm]) / AUC([DPHMar]) vs. AUC(DPMA) / AUC(DPHM) in maternal plasma suggests that at least a portion of the metabolite that is formed in the fetus is transferred across the placenta to the mother; however, the permeability appears to be substantially less than DPHM (Figure 41). If this were not the case, then the above ratios should be equivalent. Mechanisms which are involved in placental transfer of drugs may include simple diffusion, facilitated diffusion, active transport, pinocytosis, and bulk flow (Reynolds and Knott, 1989). For most drugs, simple diffusion is the dominant mechanism of transport, and therefore, blood flow and placental permeability are important in determining the rate of transfer. Placental
transfer is a function of numerous factors, including fetal and maternal plasma protein binding, lipophilicity, molecular size, pKa, blood flow, and placental morphology (Reynolds and Knott, 1989). Unlike humans, which possess a hemochorial placenta, the ovine placenta (epithelialchorial) is much less permeable to polar and higher molecular weight drugs (Rurak et al., 1991). The reason for the limited placental permeability of DPMA is not known, but may result from the large degree of protein binding, and a possible reduction in the lipophilicity of this metabolite compared to the parent drug. The pharmacological and/or toxicological implications of the prolonged persistence of DPMA in the fetal circulation are unknown at this time.

4.6.5. Fetal and Maternal Renal Clearance of DPMA and [\textsuperscript{2}H\textsubscript{10}]DPMA

There are significant differences in the extent of the renal excretion of DPMA in adult and fetal sheep. The renal clearance of DPMA was 0.9 ± 0.6 mL/min/Kg in the ewe (which corresponds to approximately 1% of the total dose), while the renal clearance of DPMA in the fetus was 0.02 ± 0.01 mL/min/Kg (~ 0.02 % of the total dose). The difference in the renal clearance of DPMA was almost 30 fold greater in the ewe compared to the fetal lamb on a weight corrected basis. In the ewe, filtration of unbound DPMA by the glomerulus would result in a renal clearance of ~0.03 mL/min/Kg, assuming that only the unbound fraction is filtered and no further reabsorption of DPMA occurs from the renal tubule. This value is 30 times less than the measured renal clearance of this metabolite, suggesting that renal secretion of this compound occurs in the adult. In the fetus, if only unbound drug were filtered, the estimate of renal clearance would be 0.020 mL/min/Kg, which is in reasonable agreement with the observed renal clearance in the fetal lamb (0.018 mL/min/Kg). Despite the fact that the fetal renal clearance
may be underestimated due to the underestimation of the fetal ΣMu, it appears that this acidic metabolite is efficiently secreted by the adult but not the fetal kidney. A similar phenomenon in fetal lambs has also been observed for other organic anions, such as para-aminohippurate, acetaminophen conjugates (sulfate and glucuronide), and morphine-3-glucuronide (Elbourne et al., 1990, Wang et al., 1986a, and Olsen et al., 1988). There are also preliminary data suggesting minimal renal excretion of valproic acid and indomethacin in fetal lambs [unpublished results]. These data suggest that while pathways for organic cation renal secretion are developed in the fetal lamb (see Section 4.6.3.), similar pathways for organic anions are not functional. This contrasts the situation observed in fetal pigs, where the excretion of PAH exceeds the inulin clearance by a factor of almost 4 (Alt et al., 1984), and in neonatal dogs in which the PAH renal secretion pathway is functional (Rennick et al., 1961, and Bond et al., 1976). However, in the rabbit the pathway develops largely during the post-natal period, and is due primarily to a post-natal increase in the intracellular content of lignin, a protein responsible for binding and intracellular storage of organic anions (Cole et al., 1978). The overall results suggest that there are species differences in perinatal development of renal secretatory pathways for organic anions.

The low renal clearance of DPMA in the fetus may help to explain the lack of measurable quantities in the amniotic fluid, since fetal urine is a major component of amniotic fluid (Battaglia and Meshia, 1986). However, renal secretion is not the only route of delivery for drugs and metabolites to the amniotic fluid. Szeto et al. (1979) demonstrated that meperidine appears in amniotic fluid despite ligation of the urethra and urachus, suggesting that drugs may be transported across the allantoic and amniotic membranes. In addition, Olsen et al. (1988) demonstrated that morphine-3-glucuronide accumulates in amniotic fluid despite complete drainage of the fetal bladder during the initial portion of the experiment in one animal. However,
a portion of the metabolite delivered to the amniotic fluid may have resulted from incomplete
drainage of urine from the fetal bladder by the catheter due to nonligation of the urethra and
urachus (Olsen et al., 1988). The reason that DPMA does not appear to traverse the fetal
membranes may be due to the very high plasma protein binding of this metabolite in fetal plasma
(see Section 3.3.2.). In contrast, the plasma protein binding of morphine-3-glucuronide is
essentially 0%, thus, it is unlikely that the transport of this conjugate is impeded by plasma
protein binding (Olsen et al., 1988). Therefore, the lack of DPMA in the amniotic fluid may
result from low quantities of the metabolite delivered to the amniotic fluid via the fetal urine, and
possible impeded transfer across the fetal membranes.

4.7. **Plasma Protein Binding of DPMA**

The plasma protein binding of DPMA in this study is extensive (~99%) in plasma
obtained from fetal and maternal sheep. DPMA is also highly bound (~97%) to human serum
albumin (Drach et al., 1970); thus, presumably DPMA also binds to fetal and maternal albumin
in sheep. Although significant differences have been noted between fetal and maternal plasma
protein binding of basic drugs thought to bind to α-1-acid glycoprotein, acidic drugs which are
known to bind to albumin show similar degrees of binding in humans (Hill and Abramson, 1988,
Kremer et al., 1988, and Vaini et al., 1991). The differences in the binding of acidic and basic
drugs in humans could result from differences in the plasma concentrations of fetal and maternal
proteins. In humans, the concentrations of α-1-acid glycoprotein are substantially lower in the
fetus, while concentrations of albumin are similar to maternal levels (Nau and Krauer, 1986, Hill
and Abramson, 1988, and Wood and Wood, 1981). Similar findings regarding the plasma
protein binding of drugs have also been reported in sheep. The binding of basic drugs appears to differ in maternal plasma and fetal ovine plasma (130-140 days gestation), for example, meperidine 75 vs. 58%, propranolol 86 vs. 50%, metoclopramide 49 vs. 39%, methadone 37 vs. 24%, lidocaine 43 vs. 27%, and DPHM 86 vs. 72% (Szeto et al., 1982c, Morgan et al., 1988, Riggs et al., 1988, Szeto et al., 1981, Kennedy et al., 1990, and Yoo et al., 1993). However, as demonstrated by the similar degree of binding of DPMA, acidic drugs appear to show similar binding to maternal and fetal plasma proteins in sheep: indomethacin 97.6 vs. 98.5%, acetylsalicylic acid 73 vs. 76%, and acetaminophen 12.5 vs. 9.3% (Anderson et al., 1980, Anderson et al., 1980a, and Wang et al., 1986a). This data suggests that sheep may be similar to humans with regard to the fetal and maternal concentrations of various plasma proteins (ovine albumin and α-1-acid glycoprotein); therefore, differences in fetal and maternal plasma protein binding could be due to the same factor in both species.

4.8. **In Vitro Metabolism of DPHM in Hepatic Microsomes Prepared from Fetal and Adult Sheep.**

Fetal drug metabolism appears to depend on species and gestational age (Juchau, 1990). In vitro drug metabolism data from small animals such as mice, rats and guinea pigs suggest a lack, or a diminished capacity for drug metabolism (Juchau, 1990, Sandberg et al., 1993, and Keunzig et al., 1974). However, evidence does exist suggesting that some of these pathways are developed in humans and higher primates by mid gestation. The presence of a fetal Cytochrome P450-3A related isoform in human fetal microsomes has been detected, and is thought to be active in the metabolism of cocaine, dextromethorphan, and ethylmorphine (Krauer and Dayer, 1991, Ladona et al., 1989, Ladona et al., 1991, and Jacqz-Aigrain and Cresteil, 1992). In
addition, there appear to be differences in the ontogeny of various fetal drug metabolizing pathways and the expression of various fetal Cytochrome P450 isoforms in humans (Juchau, 1990). From the limited data available in fetal sheep, it appears that some phase II metabolic pathways (different isoforms of glucuronyl transferase) develop prior to phase I pathways (Dvorchik et al., 1986, and Wang et al., 1986b). For example, efficient in vitro hepatic microsomal conjugation of morphine and acetaminophen occur in the fetal lamb (Dvorchik et al., 1986, and Wang et al., 1986a), and there is evidence for the in utero fetal formation of glucuronide conjugates of ritodrine, labetalol, and acetaminophen in sheep (Yeleswaram et al., 1992, Wright et al., 1992, and Wang et al., 1986b). However, N-dealkylation (i.e., Phase I) of methadone and meperidine, and the hydroxylation of benzo[a]pyrene and hexobarbital in late gestational fetal lambs occur at low rates compared to adult sheep (Dvorchik et al., 1986). In the current study, the quantities of N-demethyl DPHM (per mg of microsomal protein) formed following a 90 minute microsomal incubation were less in the fetal microsomes compared to the adult microsomes (~86% less). This appears to correlate with results obtained for the N-dealkylation of methadone and meperidine (Dvorchik et al., 1986). The reason for the diminished capacity of oxidative metabolic pathways for these substrates is not clear, but it may be due to decreased amount of the required enzymes in the fetal lamb (Cytochrome P450 isoforms). The content of Cytochrome P450 in microsomes prepared from the livers of fetal lambs is ~10 fold lower than those prepared from maternal sheep (Dvorchik et al., 1986). In the current study, the concentration of total Cytochrome P450 could not be measured in fetal microsomal preparations, while it could be estimated in preparations from maternal liver. This may have been due to both contamination of the fetal microsomal preparation with hemoglobin, and likely, the low concentration of the enzyme in the fetal microsomes. An interesting finding
in the current study is that the deamination of DPHM to form the acidic metabolite DPMA appears to proceed at equal rates in hepatic microsomes prepared from both fetal and adult non-pregnant sheep. In addition to providing direct evidence for the fetal formation of this metabolite, these results suggest that the functional in vitro capacity of this metabolic oxidative pathway is essentially equivalent to that observed in adult sheep. Recently, it has been demonstrated in our laboratory by Mr. Sanjeev Kumar that DPHM appears to be metabolized in fetal microsomal suspensions in the absence of the co-factors required for Cytochrome P450 mediated reactions (NAPH and NADPH). Furthermore, this reaction is completely blocked by the addition of pargyline (a non-specific monoamine oxidase blocker) (Dostert et al., 1989). Although monoamine oxidases (ED 1.4.3.4.: MAO) are known to be involved in the metabolism of endogenous biogenic amines, they have only recently been shown to be involved in the biotransformation of several xenobiotics (Benedetti et al., 1988). Since DPHM binds to MAOs, it is possible that the drug is also metabolized by this group of enzymes (Yoshida et al., 1989, and Yoshida et al., 1990). Thus, it is possible that the metabolism of DPHM in both mother and fetus may occur by enzyme systems other than Cytochrome P450, and that this pathway may be as efficient in fetal lambs as in the ewe. However, this hypothesis requires further study.
5. Summary and Conclusions

5.1. Synthesis of $[^2\text{H}_{10}]$DPHM, and Simultaneous Analysis of DPHM and $[^2\text{H}_{10}]$DPHM in Biological Fluids Obtained From Pregnant Sheep

The synthesis of $[^2\text{H}_{10}]$DPHM and the development of a selective and sensitive GC-MS method for the simultaneous quantitation of DPHM and $[^2\text{H}_{10}]$DPHM in biological fluids obtained from maternal and fetal sheep has been reported (Tonn et al., 1992). $[^2\text{H}_{10}]$DPHM was synthesized, and purified, and both its structure and the purity were verified. Biological samples were prepared for analysis using liquid-liquid extraction. The addition of TEA enhanced the recovery of DPHM and $[^2\text{H}_{10}]$DPHM by approximately 4 fold to yield an almost complete recovery (~100%) from the biological matrices examined. The method employed GC-MS in the electron impact ionization mode with SIM of fragment ions $m/z$ 165 for DPHM and orphenadrine (i.e., internal standard), and $m/z$ 173 for $[^2\text{H}_{10}]$DPHM. The LOQ of DPHM and $[^2\text{H}_{10}]$DPHM from a 1.0 mL sample was 2.0 ng/mL in fetal and maternal plasma, fetal tracheal fluid, and amniotic fluid. The method was validated from 2.0 ng/mL to 200.0 ng/mL for both DPHM and $[^2\text{H}_{10}]$DPHM in plasma, fetal tracheal fluid, and amniotic fluid.

5.2. Synthesis of $[^2\text{H}_{10}]$DPMA, and Simultaneous Analysis of DPMA and $[^2\text{H}_{10}]$DPMA in Biological Fluids Obtained from the Ovine Fetal/Maternal Unit

DPMA, a major urinary metabolite of DPHM in monkeys, dogs, and humans, was detected in the plasma and urine of sheep following an intravenous bolus of DPHM. In studies utilizing simultaneous administrations of both DPHM and $[^2\text{H}_{10}]$DPHM, measurement of both DPMA and $[^2\text{H}_{10}]$DPMA is required to study the disposition of this metabolite in the fetal/maternal unit. $[^2\text{H}_{10}]$DPMA was synthesized, characterized, and purified. The GC-MS
analysis method for DPMA and $[^{2}H_{10}]$DPMA utilized a single step liquid-liquid extraction procedure with toluene for sample cleanup (Tonn et al., 1995). DPMA was found to degrade under acidic conditions similar to the ones employed during the extraction of this analyte from aqueous samples. However, during the time required for extraction, degradation would result in the loss of only ~1% of the analyte. Following extraction, the samples were derivatized with N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide. A 1.0 μL aliquot of the prepared sample was injected into the GC-MS operated in the EI ionization mode and quantitated using SIM. One ion was monitored for each compound, namely, $m/z$ 165 for the internal standard, diphenylacetic acid, $m/z$ 183 for DPMA, and $m/z$ 177 for $[^{2}H_{10}]$DPMA. The ion chromatograms were free from chromatographic peaks co-eluting with the compound of interest. The calibration curve was linear from 2.5 ng/mL (LOQ) to 250.0 ng/mL in both urine and plasma. The intra-day and inter-day variabilities of this assay method were below 20% at the LOQ and below 10% at all other concentrations.

5.3. **Lack of Isotope Effects Following Fetal and Maternal $[^{2}H_{10}]$DPHM Administration**

Pharmacokinetic studies employing the simultaneous administration of both DPHM and $[^{2}H_{10}]$DPHM requires that $[^{2}H_{10}]$DPHM has the same dispositional characteristics as DPHM. That is, $[^{2}H_{10}]$DPHM should not demonstrate any measurable isotope effects. Isotope effects were not noted following equimolar intravenous doses of DPHM and $[^{2}H_{10}]$DPHM in two non-pregnant ewes. This equivalence between DPHM and $[^{2}H_{10}]$DPHM was also apparent in the cumulative amounts of DPHM and $[^{2}H_{10}]$DPHM excreted in the urine. In addition, arterial plasma concentrations of DPMA and $[^{2}H_{10}]$DPMA, and the cumulative amounts of these metabolites excreted in the urine following DPHM and $[^{2}H_{10}]$DPHM administration were
comparable. Similarly, experiments conducted following bolus administration of DPHM and $[^2\text{H}_{10}]$DPHM to fetal lambs demonstrated equivalent arterial plasma concentrations of DPHM and $[^2\text{H}_{10}]$DPHM. This equivalence was also demonstrated in amniotic and tracheal fluid, since levels of DPHM and $[^2\text{H}_{10}]$DPHM in these fluids were essentially equal. The pharmacokinetic parameters estimated showed that AUC, CL_T, Vdss, Vdβ, T1/2, and MDRT were equivalent for labeled and unlabeled drug. Following simultaneous fetal tarsal venous infusions of DPHM and $[^2\text{H}_{10}]$DPHM, the concentrations of DPHM and $[^2\text{H}_{10}]$DPHM in fetal arterial plasma, amniotic fluid, and fetal urine were equivalent. In addition, the fetal arterial plasma concentration of the metabolites DPMA and $[^2\text{H}_{10}]$DPMA were also similar. Overall, these data suggest that there are no isotope effects for the disposition of $[^2\text{H}_{10}]$DPHM in maternal and fetal sheep.

5.4. Hepatic First-Pass Metabolism of DPHM in Adult and Fetal Sheep

The administration of a 100 mg IV bolus of DPHM demonstrated that less than 1% of the dose is recovered as intact DPHM in urine. In addition, preliminary studies examining DPHM concentration in bile suggests that biliary excretion of DPHM is also not a major route of elimination, accounting for approximately 0.1% of the administered dose. These data indicate that DPHM is eliminated in adult sheep largely by hepatic or extrahepatic biotransformation. To investigate the possibility of hepatic uptake and/or biotransformation of DPHM in adult sheep and in fetal lambs, stable isotope techniques were utilized to investigate the hepatic first-pass metabolism of the drug. In adult sheep, following a total administered dose of $1.6 \pm 0.3$ mg/Kg, extensive presystemic elimination was noted, with $93.2 \pm 3.2$ % of the mesenteric dose being eliminated prior to reaching the systemic circulation. A similar experiment conducted during mild to moderate hypoxemic conditions in adult non-pregnant sheep showed that while the
extensive first-pass metabolism in three ewes remained essentially unchanged in the other two ewes the presystemic elimination decreased. These two ewes also experienced the most severe initial drop in PO₂ and O₂ saturation from normoxic levels. Other pharmacokinetic parameters, such as Vdₘ, CLₜ, and T₁/₂ appeared unaffected by hypoxemia.

Unlike adult sheep, fetal lambs receive a large portion of their hepatic perfusion from umbilical venous blood. Hence, in order to conduct a similar hepatic first-pass experiment in fetal lambs, the labeled and unlabeled drugs were administered simultaneously via the umbilical vein (fetal liver) and the lateral tarsal vein (systemic circulation). These experiments demonstrated that the fetal liver is not very efficient at extracting DPHM following umbilical venous bolus administration, since following an average dose of 3.8 ± 1.5 mg/Kg, the hepatic extraction was -10% ± 18%. A portion of the umbilical venous blood is shunted through the fetal liver via the ductus venosus (~50%). However, an extraction of DPHM from the remaining portion of umbilical venous blood that perfuses the fetal liver similar to that observed in the adult would result in ~40-45% of the umbilical venous dose being eliminated prior to reaching the systemic circulation. This was far greater than the value actually determined. To rule out saturation of fetal drug metabolizing enzymes due to rapid drug administration, simultaneous umbilical and tarsal venous infusions of DPHM and [²H₁₀]DPHM were employed. This experiment also demonstrated that the extraction of DPHM by the fetal liver following umbilical venous administration was minimal. The reason for this is not clear but may be due to a deficiency of the necessary drug metabolizing enzymes or the presence of reduced quantities of the required enzyme systems responsible for DPHM metabolism. These data also suggest that the fetal hepatic clearance does not contribute significantly towards the observed fetal non-placental clearance, while data from adult animals suggests that a significant portion of the adult
non-placental clearance may be due to hepatic elimination. However, some contribution of the fetal liver toward the fetal total non-placental clearance of DPHM cannot be ruled out.

The concentrations of oxygen and other metabolic substrates delivered to the fetus via the umbilical vein are higher in the ascending aorta than the descending aorta. A similar phenomenon has been suggested for drugs but never tested. In our studies significant differences were noted between femoral and carotid arterial concentrations of glucose, lactate, and oxygen (all being higher in carotid arterial sample). In contrast to the case with O₂ and other metabolites, the concentrations of DPHM were significantly higher in FA than in CA blood following both tarsal and umbilical venous administration, although the FA-CA difference was less with the latter route.

5.5. Simultaneous Maternal/Fetal Infusions of DPHM and [²H₁₀]DPHM

Following simultaneous fetal and maternal infusions of DPHM and [²H₁₀]DPHM, significant fetal effects were observed. There was a decrease in the intermediate voltage ECoG pattern, and an initial decrease in fetal heart rate. Fetal breathing movements tended to decrease during the infusion, but this did not reach statistical significance. The pattern of fetal effects observed appear to be intermediate to the fetal effects observed previously during separate maternal and fetal infusions of the drug. This observation appears to correlate with the intermediate fetal plasma concentrations seen in this study, compared to the previous investigation. However, a precise pharmacodynamic relationship between the constellation of fetal effects elicited by DPHM and the fetal plasma concentration of the drug remains to be defined.

In the current study, the placental and non-placental clearances of DPHM were estimated in pregnant sheep using simultaneous maternal and fetal infusions of DPHM and [²H₁₀]DPHM,
respectively. The fetal and maternal steady-state plasma concentrations were used to calculate the trans-placental and non-placental clearances of DPHM using a 2 compartment-open pharmacokinetic model. The estimates of the trans-placental clearances (CLmf and CLfm) and fetal and maternal non-placental clearances (CLfo and CLmo) agree with the estimates obtained previously through the use of time-separated infusions of DPHM to the fetus and mother. However, due to the large inter-animal variabilities, meaningful statistical inferences between the two studies were not possible.

A potential limitation of the 2 compartment-open model employed in this study is that it neglects the placenta as an organ of drug elimination and would attribute any drug metabolism by the placenta as either maternal or fetal non-placental clearance. To check this possibility for DPHM during the paired maternal-fetal drug infusions, maternal and fetal placental clearances were also estimated using the model independent Fick Method. Overall, these estimates agreed with the placental clearance values determined using the 2 compartment-open model, suggesting that significant placental metabolism of DPHM does not occur, and that the latter experimental approach is valid for the drug.

The measured fetal renal clearance of \([^{2}\text{H}_{10}]\text{DPHM}\) and DPHM in this study was 2.5 ± 0.5 mL/min/Kg. This was greater than literature values of fetal GFR, inferring that \([^{2}\text{H}_{10}]\text{DPHM}\) and DPHM are secreted by the fetal kidney. In the ewe, the renal clearance of DPHM was much lower (0.012 ± 0.005 mL/min/Kg) than reported values for GFR in adult sheep. The disparity between fetal and maternal renal clearances may be due to enhanced reabsorption of DPHM from the renal proximal tubule in the adult compared to the fetal kidney, possibly due to differences in urinary pH and urine flow rates.
Simultaneous maternal/fetal infusions of DPHM and \(^{2}\text{H}_{10}\)DPHM have provided strong evidence for the production of the deaminated metabolite of DPHM, DPMA, in both mother and fetus. DPMA and \(^{2}\text{H}_{10}\)DPMA have a much longer apparent elimination half-life in the fetus (mean ± SEM; 15.2 ± 2.5 hrs) compared to the ewe (3.0 ± 0.2 hrs). In addition, DPMA binds extensively to plasma proteins in both fetus and ewe, with greater than 99.4 and 98.9% binding, respectively. The reason for the persistence of DPMA and \(^{2}\text{H}_{10}\)DPMA in fetal blood could be due to both poorly developed elimination pathways for this metabolite and the limited placental permeability of the compound.

There were substantial differences between adult and fetal sheep in terms of the renal clearance of DPHM and DPMA. Unlike DPHM and \(^{2}\text{H}_{10}\)DPHM, the fetal renal clearance of DPMA and \(^{2}\text{H}_{10}\)DPMA \((i.e., 0.02 ± 0.01 \text{ mL/min/Kg})\) was well below the reported fetal GFR. However, in the ewe the renal clearance of these compounds was substantially greater \((0.9 ± 0.03 \text{ mL/min/Kg})\) than reported values for GFR. Thus, these data suggest that the fetal kidney can efficiently secrete DPHM (organic base), but not DPMA (organic acid). Similar findings have been noted for other organic bases \((i.e., \text{ meperidine, ranitidine, and cimetidine})\) and organic acids \((i.e., \text{ para-aminohippurate, indomethacin, and valproic acid})\). Unlike DPHM, DPMA and \(^{2}\text{H}_{10}\)DPMA could not be measured in amniotic and fetal tracheal fluid. The lack of this metabolite in amniotic fluid was not surprising since fetal urine, which contributes largely to the composition of amniotic fluid, contains very minimal amounts of \(^{2}\text{H}_{10}\)DPMA and DPMA, and further, the high degree of plasma protein binding could preclude \(^{2}\text{H}_{10}\)DPMA and DPMA from diffusing across the fetal membranes into amniotic fluid.
5.6. Fetal and Maternal Hepatic Microsomal Metabolism of DPHM

The *in vitro* metabolism of DPHM was assessed in hepatic microsomes prepared from fetal lambs and adult sheep. Hepatic microsomal incubations of DPHM resulted in the N-demethylation of DPHM in fetal and adult preparations. The amount of this metabolite formed in fetal microsomes was \( \sim 84\% \) less than that observed in adult microsomal preparations. The deaminated metabolite, DPMA, was observed following both fetal and maternal microsomal incubations, and the amounts formed were similar in both fetal and maternal microsomal incubations. This finding, in addition to demonstrating similar rates of this metabolic pathway for DPHM, provides more direct evidence for the fetal formation of DPMA.

5.7. Global Summary

In the current study, the measured fetal renal clearance of DPHM contributed only \( \sim 2\% \) to the observed fetal non-placental clearance. Further, a previous study has shown that the pulmonary extraction of DPHM is minimal \( (8 \pm 6\%) \), and since the fetal lung only receives a small portion of the fetal cardiac output \( (<5\%) \), fetal pulmonary clearance likely contributes \( \sim 8\% \) towards the fetal non-placental clearance \( (Yoo, 1989) \). Thus, the total non-placental clearance of DPHM measured by direct methods \( (i.e., \) pulmonary and renal clearance) can account for about 10\% of the observed model derived non-placental clearance estimate. If the fetal liver were to contribute the remainder of the non-placental clearance, then the extraction of DPHM from umbilical venous blood \( (i.e., \) the portion of umbilical venous blood that is not shunted past the fetal liver via the ductus venosus, \( \sim 100 \text{ mL/min/Kg} \)) across the fetal liver would have to be nearly complete. Since fetal hepatic first-pass uptake of DPHM was not consistently
detected following umbilical venous bolus and infusions of DPHM, fetal hepatic elimination of DPHM clearly does not explain the remaining portion of the fetal non-placental clearance. However, even though the fetal liver does not appear to account for a large portion of the fetal non-placental clearance, the demonstration of fetal hepatic \textit{in vitro} metabolism of DPHM \textit{(i.e.,}} fetal formation of N-demethyl DPHM and DPMA) suggests the fetal liver may be capable of metabolizing DPHM, and thus still contribute a portion of the fetal non-placental clearance.

While the low renal clearance of intact DPHM is similar both in fetus and mother, there appears to be a large difference in the hepatic uptake and/or metabolism of DPHM between mother and fetus. This suggests that the pathways for the non-placental elimination of DPHM differ in mother and fetus.

It is still not known which fetal organ(s) are primarily responsible for the observed fetal non-placental clearance. Acetaminophen exhibited similar characteristics since only 38\% of the CLfo has been accounted for, even though a much larger portion (~98\%) of the maternal non-placental clearance could be explained by maternal renal elimination and conjugation reactions.

An analogous situation was also observed for ritodrine, where only ~22\% of the fetal dose could be accounted for by glucuronide conjugation. Since we can only account for a small portion of fetal drug elimination, our understanding of fetal non-placental metabolism and drug disposition remains limited and requires substantial further study. Assessment of the quantitative role of DPMA, which is formed both \textit{in utero} by the fetal lamb and \textit{in vitro} by fetal hepatic microsomes, may account for a further portion of the fetal non-placental clearance. It would also be prudent to investigate the role of other identified and as of yet unidentified metabolites of DPHM towards the fetal and maternal non-placental clearances of this drug.
5.8. Conclusions

The conclusions which can be drawn from the experiments carried out in this thesis are:

1. There are no isotope effects which would interfere with the interpretation of the results from experiments conducted using simultaneous administrations of both DPHM and $[^{10}H_10]$DPHM in non-pregnant, pregnant, and fetal sheep.

2. There is a considerable hepatic first-pass drug uptake in adult sheep following mesenteric administration of DPHM.

3. There is no significant fetal first-pass effect following umbilical venous bolus DPHM administration and infusion, suggesting that this mechanism does not play a major role in reducing the fetal exposure to maternally derived DPHM. However, due to the variability associated with these results and the in vitro data from the fetal hepatic microsomal incubations, fetal hepatic elimination of DPHM cannot be completely ruled out.

4. Fetal renal clearance of DPHM in both maternal and fetal lambs cannot account for a large portion of the measured non-placental fetal (~2%) or maternal clearance (~0.1%).

5. While the fetal kidney appears to be able to readily excrete DPHM (i.e., renal clearance is greater than literature values of fetal GFR), this is not the case for DPMA.
6. The metabolism of DPHM to DPMA occurs both in vitro (hepatic microsomes) and in vivo by fetal lambs and adult maternal sheep.

7. DPMA persists longer in fetal sheep than in the mother.

8. N-demethyl DPHM is formed in vitro both in fetal and adult hepatic microsomes, but the formation by fetal hepatic microsomes in much lower than in the adult.
6. References


Smith, K. J., Wade, T. A., Martin, T. J., Chen, X. Y., and Simons, F. E.:


Szeto, H. H., Clapp, J. F., Larrow, R., Hewitt, J., Inturrisi, C. E., and Mann, L. I.:

Szeto, H. H., Kaiko, R. F., Clapp, J. F., Larrow, R. W., Mann, L. I. and Inturrisi, C. E.:

Szeto, H. H., Mann, L. I., Bhakthavathsalan, A., Liu, M. and Inturrisi, C. E.:


Yeleswaram, K., Rurak, D. W., Kwan, E., Hall, C., Doroudian, A., Wright, M. R., Abbott, F. S. and Axelson, J. E.: Transplacental and nonplacental clearances,


Appendix 1: Experimental animal data and experimental details

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<th>MW (kg)</th>
<th>FW (kg)</th>
<th>GA (days)</th>
<th>No. of fetuses</th>
<th>Sex</th>
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MW-maternal weight, FW-fetal weight at time of experiment, GA-fetal gestational age at time of experiment. na-not applicable. MV-maternal femoral vein, Mes-maternal mesenteric vein, TV-fetal lateral tarsal vein, UV-common umbilical vein.

F. Simultaneous fetal umbilical and tarsal venous infusion study |

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<td>[^{2}H_{10}]DPHM-UV DPHM-TV</td>
<td>60.8 60.6</td>
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<td>75.4 79.2</td>
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MW-maternal weight, FW-fetal weight at time of experiment, GA-fetal gestational age at time of experiment. na-not applicable. MV-maternal femoral vein, Mes-maternal mesenteric vein, TV-fetal lateral tarsal vein, UV-common umbilical vein. *Ewe died in utero weight determination based on time of death
<table>
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<tr>
<th>EWE No.</th>
<th>MW (kg)</th>
<th>FW (kg)</th>
<th>GA (days)</th>
<th>No. of fetuses</th>
<th>Fetal Sex</th>
<th>Administration Route</th>
<th>Dose ug/min</th>
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<td>127</td>
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</table>
APPENDIX 2

$^1$H-NMR (400 MHz) of Diphenhydramine HCL in D$_2$O
APPENDIX 2 (Cont.)

$^1$H-NMR (400 MHz) of [H$_{10}$]Diphenhydramine HCL in D$_2$O
APPENDIX 2 (Cont.)

$^1$H-NMR (200 MHz) of $[^2$H$_{10}]$Diphenylmethoxyacetic acid in CDCl$_3$

Note TMS was used as an internal standard in this sample
APPENDIX 2 (Cont.)

$^1$H-NMR (200 MHz) of Diphenylmethoxyacetic acid in CDCL$_3$
APPENDIX 3: Diagram showing sites of catheter placement in the fetal lamb